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# **ANNALES MEDICINAE EXPERIMENTALIS ET BIOLOGIAE FENNIAE**

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## FOETAL OVARY AND TESTIS

### HISTOLOGICAL AND HISTOCHEMICAL OBSERVATIONS

by

HANNES SAURAMO

(Received for publication October 22, 1960)

The present work was carried out in an effort to continue my earlier studies on the ovary of the embryonic and foetal period (3). For comparison the testis has also been studied with special reference to the interstitial tissue, this article being at the same time an introduction to further studies of the ovarian interstitial cells.

#### MATERIAL AND METHODS

The series consisted of 24 cases. Four of this number were early cases, crown-rump length from 3 to 8 mm., age 3.5–5.5 weeks. Two cases were in the gonadal stage, crown-rump length 11–15 mm., age 6–6.5 weeks. The testes were studied in 8 cases, crown-rump length 25–180 mm., age from 8 weeks to 6 lunar months. Finally there were 10 cases of ovaries, crown-rump length 50 mm. to newborn, age 2.5 to 10 lunar months.

The fixing agents were 10 per cent formol and Allen's P.F.A.<sub>3</sub>. The following staining methods were used: 1. Ehrlich Haematoxylin-Nuclear Fast Red, 2. Weigert's Iron Haematoxylin-van Gieson stain, 3. Periodic Acid-Schiff's (PAS) reagent method (for polysaccharides), 4. Feulgen's stain (for deoxyribonucleic acids, DNA), and 5. Diastase-PAS. — The DNA content refers to the intensity of the Feulgen reaction.

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Supported by a grant from the Sigrid Jusélius Foundation.

## RESULTS

## EARLY EMBRYONIC PERIOD

Crown-rump length from 3 to 8 mm.: Gonad not developed.

Crown-rump length from 11 to 15 mm.: Gonadal epithelium and some mesenchyme is seen. No gonadal differentiation has occurred. PAS-positive substances are seen especially in the epithelium. The Feulgen reaction is distinct throughout.

## TESTIS

25 mm.: Surface epithelium, sex cords and connective tissue are present. The interstitium is not yet typical and there is no tunica albuginea. PAS-positive substances occur in the surface epithelium and the Sertoli cells, elsewhere they are of slight amount. The Feulgen reaction is seen in all nuclei, particularly in nuclei of mesenchyme cells.

45 mm.: A thin tunica, sex cords, developing seminiferous tubules and an abundance of interstitial cells are seen. PAS-positive substances occur in the tunica, in the Sertoli cells clearly, and in the interstitial cells to some extent. The Feulgen reaction is distinct throughout, especially in the nuclei of connective tissue cells.

70 mm.: A tunica, solid seminiferous tubules, sex cords, and numerous interstitial cells are seen. There are PAS-positive substances in the cytoplasm of the interstitial cells, to a less extent in tubules. The Feulgen reaction is distinct in the tubules, weaker in interstitial cells.

100 and 110 mm.: A distinct tunica, solid tubules, sex cords, and numerous interstitial cells are seen. PAS-positive substances are present to some extent in Sertoli cells and interstitial cells, little if at all in spermatogonia. The Feulgen reaction is distinct in the tubules and of course in the nuclei of connective tissue cells.

150 mm.: A tunica, solid tubules, a small number of sex cords and interstitial cells in great abundance occur. PAS-positive substances are seen in the cytoplasm of the interstitial cells, but not to any extent in the tubules. The Feulgen reaction is obtained in the tubules, not appreciably in the interstitial cells.

170 mm.: A fairly thick tunica is seen, also densely packed solid tubules and a considerable number of interstitial cells. PAS-positive

substances occur in small amount. The Feulgen reaction is of a corresponding kind.

180 mm.: The tunica is thick, there are solid tubules and numerous interstitial cells. PAS-positive substances are seen to some extent in the tubules and interstitial cells. A Feulgen reaction is obtained in the tubules, but scarcely at all in the interstitial cells.

*Summary.* — The surface epithelium becomes gradually thinner, losing its importance. The tunica albuginea is distinct in the 45 mm. foetus, but thin. It thickens gradually. The sex cords are gradually replaced by the seminiferous tubules, which are solid. In the interstitium there is at first mesenchyme between the sex cords, but soon there are numerous interstitial cells. PAS-positive substances occur at first in the surface epithelium and the Sertoli cells, later to some extent also in the cytoplasm of the interstitial cells, but then they weaken again; in spermatogonia they are weak. The Feulgen reaction is distinct throughout at first, later it weakens in the interstitial cells.

*Conclusions.* — A foetus of 45 mm. at least has a tunica in the testis, the formation of solid tubules begins, Sertoli cells and spermatogonia are differentiated, and interstitial cells are distinctly present. PAS-positive substances occur from the outset in the Sertoli cells, in spermatogonia not appreciably, and in the cytoplasm of interstitial cells to some extent. Evidently the spermatogonia and interstitial cells are in some kind of latent phase. In a 45 mm. foetus the three typical cell types of the testis are already developing, *i.e.* spermatogonia, Sertoli cells, and interstitial cells.

#### OVARY

50 mm.: Ova are present. PAS-positive substances occur to some extent throughout, more in the superficial portions. The Feulgen reaction is of a corresponding kind.

80 mm. (2): As above.

85—90 mm. (3): Ova and PAS-positivity as above. The Feulgen reaction is distinct in the germinal epithelium, and similarly in the nuclei of connective tissue cells, but weak in the ova.

170 and 180 mm.: Surface epithelium, ova, primordial follicles and small growing follicles are seen. PAS-positive substances occur in fairly small amount, especially in the deeper portions. A follicle system in process of degeneration may show PAS-positive sub-

stances distinctly. The Feulgen reaction is strong in the germinal epithelium, distinct in the surface parts and the connective tissue, but weak in follicle ova.

Newborn (2): There are also atretic follicles. PAS-positivity is weak elsewhere but not in the connective tissue. The Feulgen reaction is distinct in the connective tissue, in the follicle epithelium and germinal epithelium.

*Summary.* — The surface epithelium is preserved in the form of germinal epithelium. There is no tunica. Different stages of the follicle system appear gradually. The interstitium consists of connective tissue of ordinary appearance. PAS-positive substances seem to occur at first distinctly; weakening seems to take place last in the superficial parts, and in the newborn only the connective tissue shows these substances distinctly. True, they may occur in connection with degenerating follicles and may be distinct. The Feulgen reaction is more definite at first; later, in ova in particular, it does not occur to any extent.

*Conclusions.* — The surface epithelium is preserved as a germinal epithelium. It seems that only the germinal epithelium is in a functioning stage proper. The ova and the follicle system are in some kind of latent phase. Nothing indicates that the ova do not arise from the outset in the ovary itself and develop there. The follicle epithelium develops in a 19–20 cm. foetus, and at about 28–30 cm. the Graafian follicles, *i.e.* the granulosa and theca, are already seen.

#### CONTROL STUDY

It has been demonstrated with the diastase-PAS method that the usually fairly weak PAS-positivity does not weaken much. Such a weakening is most clearly seen in Sertoli cells. Thus only the Sertoli cells contain glycogen in appreciable amount. The diastase-fast PAS-positive substances must be understood to be glycoproteins. The bowel, for instance, here serves for comparison: it occurs in many specimens of the foetal cross-section. The bowel shows very strong PAS-positivity, and in part this is accounted for by diastase-fast substances. Probably these are glycogen and mucoproteins.



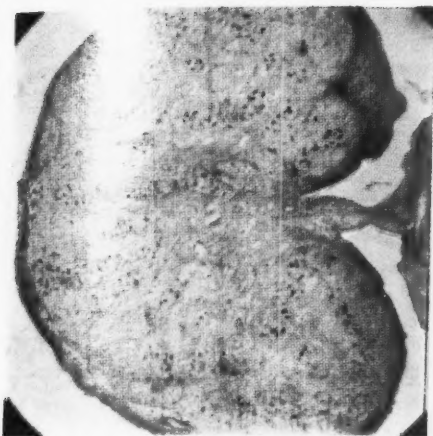


Fig. 1. — Early foetal testis. PAS-positive substances in the Sertoli cells. PAS, +100.

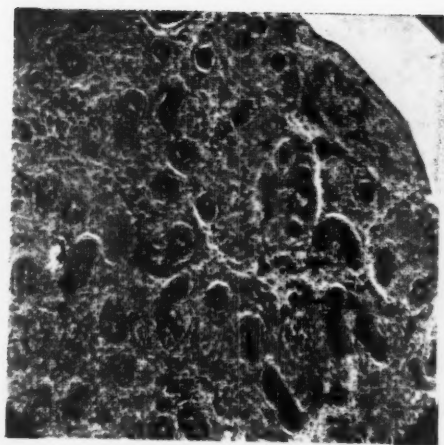


Fig. 2. — Foetal testis. Interstitial cells, and tubules. + 100.

Fig. 3. — Foetal testis. Interstitial cells, and tubules. + 100.

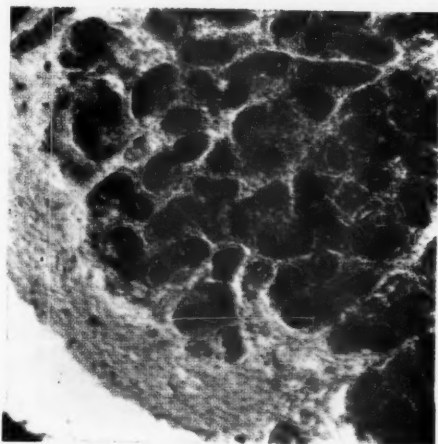
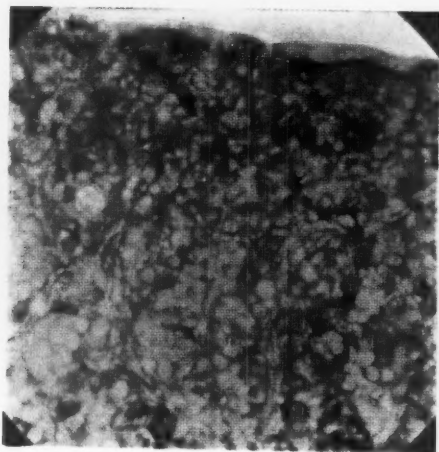
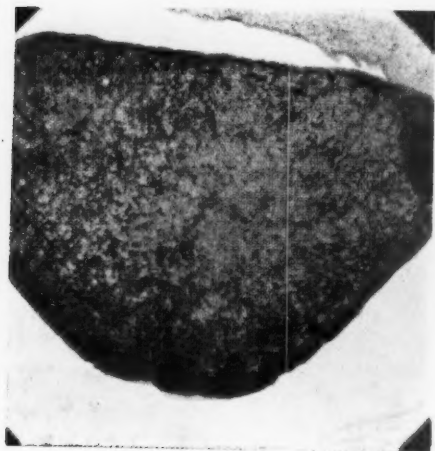
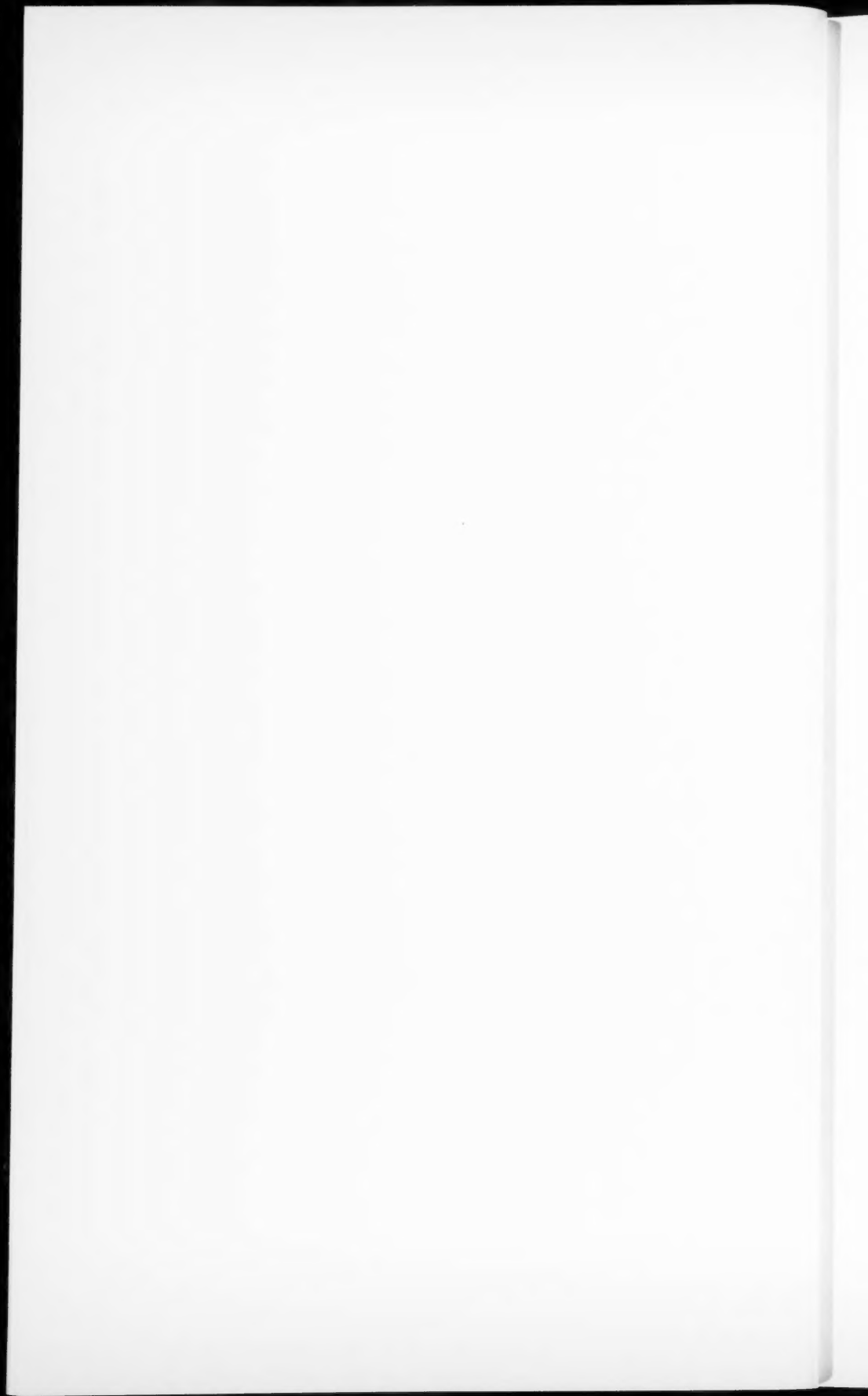


Fig. 4. — Early foetal ovary. PAS-positivity is moderate. PAS, + 100.

Fig. 5. — The same case. Surface portion. PAS-positive substances. PAS, + 450.





## DISCUSSION

A repetition of detailed histological descriptions is not called for here (3, 4, 5). A histochemical observation worth noticing is that the primordial germ cells possess a very high alkaline phosphatase activity (2). A Schiff positive substance has been found in the granulosa layer of the Graafian follicle, associated with activity of the foetal ovary in cases of toxæmia of pregnancy (1). In the testis, finely divided glycogen is seen in the spermatogonia and in the cell plasma of Sertoli cells, but not in the Leydig interstitial cells although these contain PAS-positive unknown polysaccharides or glycoproteins (4).

## SUMMARY

The three typical cell forms of the testis, *i.e.* the spermatogonia, the Sertoli cells and interstitial cells, are in process of developing at the beginning of the foetal period in the third lunar month. In the foetal ovary, the follicle epithelium develops in the fifth lunar month and the granulosa and theca in the sixth lunar month at the earliest.

PAS-positive substances are distinctly present in the testis of the embryo and foetus in Sertoli cells, to some extent in interstitial cells, and in slight amount in spermatogonia. Correspondingly the ovary contains some amount of PAS-positive substances principally in the germinal epithelium and the primordial ova close to the germinal epithelium. There is little glycogen, chiefly in the Sertoli cells. The other PAS-positive substances are evidently glycoproteins, and they are most distinctly seen in the Sertoli cells. The Feulgen reaction is weak in the interstitial cells of the testis and fairly weak in spermatogonia and oogonia and in follicle ova. It seems that in the testis the spermatogonia and the interstitial cells and in the ovary the oogonia and the follicle system, are in a latent phase. In the ovary the germinal epithelium is functioning. The effect of chorionic gonadotropin (ICSH) appears in the epithelium of the Graafian follicle (the granulosa and theca) in the ovary and in the Sertoli and interstitial cells of the testis during intra-uterine life.

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FROM THE CENTRAL HOSPITAL OF SOUTHERN SAIMAA, GYNAECOLOGICAL  
AND OBSTETRICAL DEPARTMENT, LAPPEENRANTA

## CYTOTROPHOBLAST OF THE PLACENTA AND FOETAL MEMBRANES IN NORMAL AND PATHOLOGICAL OBSTETRICS

by

HANNES SAURAMO

(Received for publication October 22, 1960)

There are a variety of cytotrophoblastic structures in the placenta and they differ in characteristics. The purpose of this study was to deal with these structures mainly during the third trimester of pregnancy. However, for an understanding of the occurrence of cytotrophoblastic structures, the placenta and the foetal membranes during the first and second trimesters must also receive attention. Nor can the syncytiotrophoblast be entirely disregarded.

### MATERIAL AND METHODS

The study was based on 75 normal and pathological cases. The removal of tissue specimens, fixation, and histological and histochemical methods have been described in earlier studies (3, 4).

### RESULTS

Excluding the earliest stage in the development of the placental and foetal membranes, the later cytotrophoblastic structures can be divided into several groups:

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<sup>1</sup> Supported by a grant from the Sigrid Jusélius Foundation.

|                 |                         |
|-----------------|-------------------------|
| Cytotrophoblast | of the chorionic plate  |
| »               | of the placental septa  |
| »               | of the chorionic villi  |
| »               | of the basal plate      |
| »               | of the placental border |
| »               | of the chorion laeve    |

The characteristics of especially the cytotrophoblastic structures in the placenta and foetal membranes have been studied in the following groups of cases:

normal term placenta  
immature, early and late premature placenta  
placenta in toxæmia of pregnancy  
placenta in diabetes  
placenta in blood group incompatibility  
placenta in hydramnion  
over-term placenta

The most important features of the cytotrophoblastic structures and also of the decidua will be described below.

#### HISTOLOGICAL AND HISTOCHEMICAL STUDY

Normal term placenta and membranes show subchorial connective tissue degeneration, even necrosis. The connective tissue of the decidua is often abundant. PAS- (periodic acid-Schiff) positive substances occur to some extent, only in the cells of the large cytotrophoblast structures, especially in the chorionic plate. In greater amount, however, they are present in the chorion laeve, *i.e.* in the membranes: here they occur to some degree, in places in considerable amounts. The decidual cells may contain a small amount of PAS-positive substances. In normal term placenta there is slight (or moderate) chorionic and decidual degeneration.

In the cases of immature and premature placenta and foetal membranes without apparent cause, the degenerative changes are strong (or moderate). In immature cases there is strong degeneration and the chorion laeve is thin. Decidual necrosis is strong. PAS-positive substances are of only small amount or absent in the cells of the chorion laeve. In early premature placenta, degeneration

is moderately strong. The cells of the chorionic plate may sometimes show a small amount of PAS-positive substances. The chorion laeve is also in these cases in a state of strong degeneration, thin, and shows areas of necrosis. The decidua shows a similar picture. PAS-positive substances may occur in places in the cells of the chorion laeve or may be absent. In late premature placenta the degenerative changes are weakest. In the chorionic plate PAS-positive substances are sometimes definitely present. The chorion laeve is also here thin and in places there is connective tissue in abundance and necrosis. Decidual degeneration is also of moderate degree. In the chorion laeve PAS-positive substances occur definitely, in slight amount, or are absent. The cells of the decidua may at times show a small amount of these substances. Strong or moderate chorionic and decidual degeneration is seen in immature and premature placenta.

In toxæmia of pregnancy different degenerative changes are seen in the placenta and membranes depending upon the type of toxæmia. The cells of the chorionic plate may show a small amount of PAS-positive substances. The chorion laeve is thin with degeneration and may contain small amounts of PAS-positive substances, in places definitely. The decidua in particular has undergone degeneration. Thus toxæmia is associated with strong chorionic and decidual degeneration, especially the latter.

In diabetes there is degeneration in the placenta and membranes; the amount of connective tissue and the presence of glycogen are related to the severity of the disease and to treatment. PAS-positive substances occur to some extent in the chorionic plate, distinctly in the chorion laeve and decidua. Chorial and decidual degeneration is slight in the cases properly treated.

In the case of blood group incompatibility the placenta and membranes show changes which are also entirely related to the nature of the case, as far as degeneration, amount of connective tissue and presence of glycogen are concerned. The decidua is preserved. In mild cases there are PAS-positive substances in the chorionic plate, the placental septa, and the chorion laeve. Cases of slight blood group incompatibility show weak chorial and decidual degeneration.

In cases of hydramnion there are strong degenerative changes in the placenta and membranes. In the membranes the chorion



laeve is fairly thick but degenerated. The decidua is in a state of strong degeneration. There may be a small amount of PAS-positive substances but only in the cells of the chorion laeve. Chorial and decidual degeneration is strong in cases of hydramnion.

Over-term placenta and membranes show slight degenerative changes in mild cases, corresponding roughly to normal term cases. More severe cases are scarcely seen at present in obstetric wards, because the cases are treated in time. PAS-positive substances may occur in small amounts in chorionic structures. The chorion laeve and decidua are fairly well preserved and degeneration is slight.

#### CYTOTROPHOBLASTIC STRUCTURES

The chorionic plate of normal term placenta shows slight degenerative changes. PAS-positive substances in the cells diminish continually towards term. The deposition of fibrin increases. In late premature placenta the chorionic plate is of almost the same kind. The chorionic plate is fairly well preserved in mild toxæmia, mild diabetes, slight blood group incompatibility, and in slight postmaturity. In the other cases the changes are strong.

The cytotrophoblast of the placental septa (of the anchoring villi) often contains clusters of cytotrophoblastic cells. The changes are on the whole similar to those in the chorionic plate. PAS-positive substances, however, occur in cytotrophoblastic cells in slight amount and irregularly. They are distinctly present in slight blood group incompatibility only.

The cytotrophoblast of the basal plate consists of scattered large cells. Some amount of PAS-positive substances are seen, mainly in the cytotrophoblast cells of the decidual tissue (placental site cells). To some extent they are also present in normal term placenta, late premature placenta, and in mild diabetes. These substances are usually absent in cases in which the decidua has undergone strong degeneration.

Cytotrophoblast cells of the chorionic villi (Langhans cells) occur only to a slight extent during the third trimester (2). In normal term placenta cytotrophoblast cells are seen here and there in chorionic villi. In premature placenta a few cytotrophoblast cells occur in the villi. Over-term placenta usually shows no cytotropho-



Fig.  
or



Fig.  
plate



Fig. 1. — Normal term membranes. Slight or moderate PAS-positivity. Alcian blue-PAS, + 100.



Fig. 2. — The same case. Chorionic plate in the border of the placenta. Moderate PAS-positivity. Alcian blue-PAS, + 100.



Fig. 3. — The same case. Placental chorionic plate. No PAS-positive substances. Alcian blue-PAS, + 100.

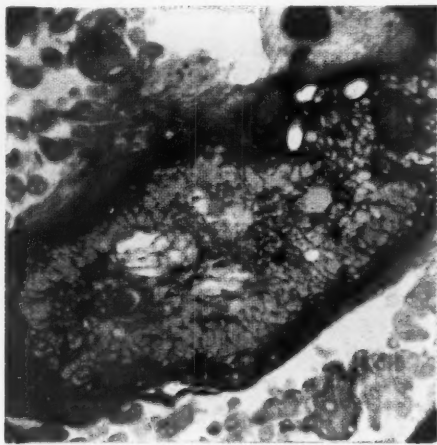


Fig. 4. — The same case. Placental septum. Weak PAS-positivity. Alcian blue-PAS, + 100.

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blast cells. There are more of these cells in borderline cases to post-maturity. In toxæmia of pregnancy these cells are few. There are no PAS-positive substances.

The cytotrophoblast of the placental border is exceptional in that the different tissues unite here, *i.e.* the placental chorionic plate and decidua, and the foetal membranes; in addition there is the sinus marginalis. The deposition of fibrin is extremely strong and the chorionic villi, fairly far apart, are frequently covered by fibrin. The most typical ischemic villi in fact occur at the placental border. At the same time there are cytotrophoblastic structures, which may sometimes be large, in this very area. The degenerative changes are very strong and the microscopic picture is relatively disrupted. It has been claimed that regeneration of cytotrophoblast cells occurs in ischemic villi (1), and also that cytotrophoblast cells of ischemic villi contain diastase-fast PAS-positive substances (5). In the presence of ischemic necrosis, this shows the picture of a coagulation necrosis. The outlines of cells persist for a long time. Deposition of fibrin is strong, and the connective tissue shows strong hyaline degeneration. The PAS-method yields an intense staining of many structures, which are perhaps more distinct still with the diastase-PAS method. It is possible that we are not here concerned with something other than strong degeneration. Deposition of glycogen can occur.

The cytotrophoblast of the chorion laeve is interesting from the point of view of function. Subchorial connective tissue degeneration, even necrosis, is already seen in normal term membranes. The degree of degeneration in the chorion laeve is in agreement with what has been said above of the different disease groups. PAS-positive substances occur in normal membranes to a slight extent or in places considerably. These substances can be more prominent in diabetes and blood group incompatibility only, in the other cases they are of less amount.

#### SUMMARY

The study was based on 75 cases of placenta and foetal membranes, normal and pathological.

Disregarding the mildest forms of the various diseases, it can be said that the placenta and foetal membranes in all of them

show degenerative changes which are stronger than in normal term placenta and foetal membranes.

Histologically the degenerative changes are similar but vary in degree.

Histochemical studies reveal greater differences, yet — with the methods used — there seem to be comparatively few specific changes.

The cytotrophoblastic structures can be divided into several groups. It seems, however, that at the end of pregnancy and especially in normal term placenta and membranes, there are two main groups. The chorion laeve of the membranes and the chorionic plate of the placental border make up one group. The second group, with different characteristics, consists of the other cytotrophoblastic structures in the area of the placenta proper. The impression is conveyed that the former structures have charge mainly of hormonal function — the production of chorionic gonadotrophin. The latter structures form part of the placenta proper including the chorionic villi; since the placenta has a filtering function the hormonal activity may be slighter. In other words, it may be assumed that the chorionic cytotrophoblast of the foetal membranes plays a more important part during the latter half of gestation than has been earlier supposed.

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## SUPPLEMENTARY COMMENT ON A TRYPSIN DETERMINATION METHOD

by

T. VARTIO

(Received for publication October 25, 1960)

In the trypsin determination method described earlier by the present writer (3) the substrate used was dried human serum. As this substrate as such, also in dried form, could contain some amounts of antitrypsins (2), the present writer has proved, in some cases, the method with undenaturated and denaturated substrate. The denaturation is performed according to Gowenlock (1): The undenaturated substrate solution is made up to contain 4 g. of dried serum in 100 ml. of 0.01 N NaOH solution and its pH is about 10. This solution, contained in a flask plugged with cotton wool, is immersed in a boiling-water bath for 12 min. After this time, the flask is transferred to a bath of running cold water to cool to room temperature and the resultant solution is filtered through cotton wool. To the filtrate is added 0.25 vol. of the  $\text{NH}_4\text{Cl-NH}_4\text{OH}$  buffer solution. The pH of the substrate solution should be 9.5.

The results were as follows. The determinations were made in triplicates. The proteolytic activity of the serum was expressed in mg:s of human serum protein broken up in an hour by one ml. of the specimen to be studied, and the proteolytic activity of the urine was expressed in mg:s of human serum protein broken up in an hour by the amount of urine excreted in an hour:

|                                       | Trypsin                 |             |                           |             |
|---------------------------------------|-------------------------|-------------|---------------------------|-------------|
|                                       | Serum<br>Mg. Protein/MI |             | Urine<br>Mg. Protein/Hour |             |
|                                       | Un-<br>denaturated      | Denaturated | Un-<br>denaturated        | Denaturated |
|                                       |                         |             |                           |             |
| Age Sex                               |                         |             |                           |             |
| 34 m duodenal ulcer . .               | —                       | —           | 3.7                       | 4.8         |
| 52 » gastric ulcer . . . .            | 0.00                    | 0.27        | 2.1                       | 6.5         |
| 65 f pernicious tape-<br>worm anaemia | 0.18                    | 0.20        | 2.4                       | 2.4         |
| 42 m acute pancreatitis               | 0.12                    | 0.22        | 2.1                       | 7.9         |
| 76 f » »                              | 0.18                    | 0.20        | 1.2                       | 1.8         |
| 34 m » »                              | 0.12                    | 0.15        | 1.3                       | 1.5         |
| Mean                                  | 0.12                    | 0.20        | 2.1                       | 4.1         |

Thus, on an average, the serum trypsin values were 0.08 mg. higher when denaturated substrate was used, and the urine trypsin values correspondingly 2.0 mg. higher.

#### SUMMARY

In the trypsin determination method, described by the present writer, undenaturated and denaturated dried human serum was used as substrate. On an average, the serum and urine trypsin values were somewhat higher when denaturated substrate was used, which could be a consequence of the fact that dried human serum undenaturated contains some amounts of antitrypsins.

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## IMMUNIZATION WITH ECHO VIRUSES GROWN IN MONKEY KIDNEY CELL CULTURE

SPECIFIC AND NON-SPECIFIC RESPONSES OF MICE, GUINEA PIGS,  
AND RABBITS

by

PEKKA HALONEN<sup>1, 2</sup>

(Received for publication August 15, 1960)

Various non-specific activities, such as cytotoxicity, non-specific «neutralization», and host complement-fixing (CF) activity, could be noted in viral antisera prepared by immunizing the animals with cell culture grown virus and similar effects have been found in anti-cellular sera (2, 3). During the course of an enterovirus study difficulty was encountered in preparing specific antisera to certain ECHO viruses even with prolonged hyperimmunization of rabbits. When these antisera were tested in low dilutions they showed non-specific activities. This prompted a study of the development of specific virus antibody activity and non-specific activity in ECHO virus antisera prepared in various animals according to various immunization schedules.

This report will present data on the homologous neutralizing and CF antibody titers, nonspecific, heterotypic «neutralization» titers, cytotoxicity and host CF activity in sera of mice, guinea pigs and rabbits immunized with monkey kidney cell culture grown ECHO viruses.

<sup>1</sup> Aided by a grant of Sigrid Jusélius Foundation.

<sup>2</sup> The early experiments reported in this paper were performed while working in the Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, N.I.H., Bethesda, Md., U.S.A.

## MATERIALS AND METHODS

*Virus Strains.* — Prototype strains of ECHO types 4, 5, 6, 11, 12, 13, and 14 (4, 5) were used to prepare immunization and CF antigens, and in neutralization tests.

*Cell Cultures.* — Monkey kidney cell cultures were grown in a medium containing 2% calf serum, 0.5% lactalbumin hydrolysate, and 97.5% Earle's solution. At the time of inoculation the cultures were washed twice with Hanks' solution and 1.0 ml of medium no. 199 was added to tube cultures and 40 ml to 32 oz. prescription bottle cultures.

*Immunization Antigens.* — The immunization antigens were prepared from monkey kidney cell culture grown virus. After complete degeneration had taken place, the bottles were frozen and thawed once, and the fluids were then harvested and centrifuged at 3,000 r.p.m. for 15 minutes. The supernatant was collected, and stored at  $-20^{\circ}\text{C}$  until used as unconcentrated immunization antigen. For the preparation of concentrated antigens the harvested fluids were centrifuged in a Spinco at 30,000 r.p.m. for 3 hours, the pellet was resuspended to  $10 \times$  concentration in medium no. 199, and the suspension was treated in a Raytheon sonic oscillator for 3 minutes at 10 kilocycles and 1.25 amperes. All antigens containing penicillin were treated with penicillinase before use in the immunization of guinea pigs.

*CF Antigens.* — Fluorocarbon-treated and untreated CF antigens were prepared as reported previously (6, 7). A «normal» cell culture antigen for testing anti-host activity of antisera was prepared by inoculating a monkey kidney cell culture with Coxsackie B type 5 virus.

*CF Test.* — Complement fixation tests were performed by the Bengtson technique (1) with some modifications (6).

*Infectivity Titrations and Neutralization Tests.* — When infectivity titrations were carried out, 4 cell culture tubes were used per 10-fold dilution. Titers were calculated by the Reed-Muench method (8) and expressed as TCID<sub>50</sub> per 0.1 ml. Neutralization tests were made by mixing 100 TCID<sub>50</sub> of virus with series of 2- or 4-fold dilutions of serum. After 1 hour at room temperature each mixture was inoculated into two cell culture tubes.

## RESULTS

It had previously been found that with certain ECHO types it was difficult even by prolonged immunization of rabbits to prepare antisera which would neutralize the homotypic virus even in low serum dilutions, whereas some other ECHO types produced antisera with high neutralization titers after a short period of immunization. Hence, for the first series of immunization studies ECHO type 6 virus was selected to represent a good antigen and ECHO types 4 and 14 were chosen as poor immunization antigens. The ECHO type 6 antigen used throughout in this study was

unconcentrated, and it had an infectivity titer of 7.6. ECHO type 4 and 11 antigens were concentrated by the method indicated above. They had infectivity titers of 6.0 and 6.3.

#### DEVELOPMENT OF HOMOTYPIC NEUTRALIZING ANTIBODY TITERS

*Mice.* — 50 mice weighing 16–18 gm were immunized weekly with 5 doses of 0.25 ml of unconcentrated ECHO type 6 virus given intraperitoneally. The sixth dose was given 9 weeks after the fifth. Ten mice were decapitated 1, 2, 3, 4, and 16 weeks after the first immunization, and their blood was collected and pooled. The ECHO type 6 neutralizing antibody titers of the serum pools was estimated in a single neutralization test. The highest neutralization titer after the series of repeated immunizations was 1:256 (Fig. 1). One week after the 15-week booster dose it was 1:512.

Two other groups of mice were immunized with ECHO type 6 virus, one with two doses of 0.25 ml given intraperitoneally at an interval of 12 days and bleeding 4, 8, and 12 days after the last immunization, the other with two doses of 0.25 ml, the first intravenously, the second intraperitoneally, at an interval of 7 days, and bleeding 4 to 8 days after the last immunization. In both groups the homotypic neutralizing antibody titers were 1:32 or less.

Two groups of mice were immunized with 4 doses of 0.25 ml of concentrated ECHO type 4 virus given intraperitoneally at successive intervals of 12 days, 7 days, and 9 weeks. In one group the first dose was given with Freund's complete adjuvant (9), the second and the third with Freund's incomplete adjuvant, and the fourth without adjuvant. In another group no adjuvant was used. Homotypic neutralizing antibodies could be found only after the booster dose at 12 weeks, when the titers were 1:32 in the group with adjuvant and 1:8 without adjuvant (Fig. 1).

*Guinea Pigs.* — Four groups of guinea pigs were immunized with ECHO type 6 virus. The various immunization schedules and doses and the development of homotypic neutralization titers in each group are shown in Fig. 2. The highest titers were in group IV, in which the highest level (1:32, 768) was reached after two doses with Freund's complete and incomplete adjuvants given 19 days apart. Low titers were found in group I, in which the animals were immunized with 5 weekly doses.

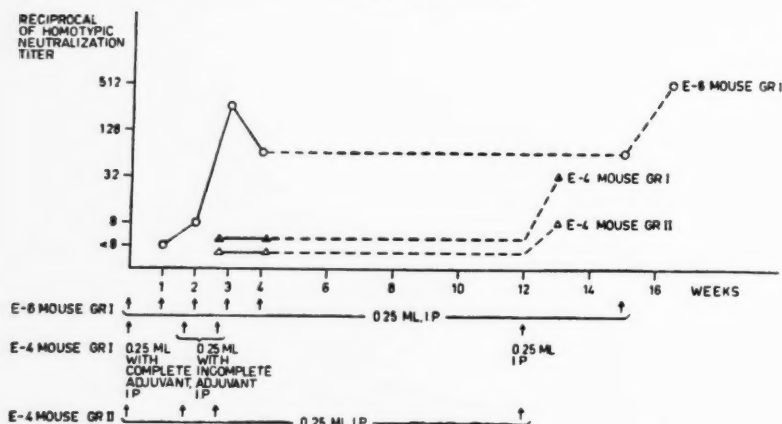


Fig. 1. — Development of homotypic neutralizing antibody titers in a group of mice immunized with unconcentrated ECHO type 6 virus (E-6, mouse group I), and two groups of mice immunized with concentrated ECHO type 4 virus (E-4, mouse groups I and II). Immunization of animals indicated by arrows. Each point represents the titer of pooled sera of 10 animals.

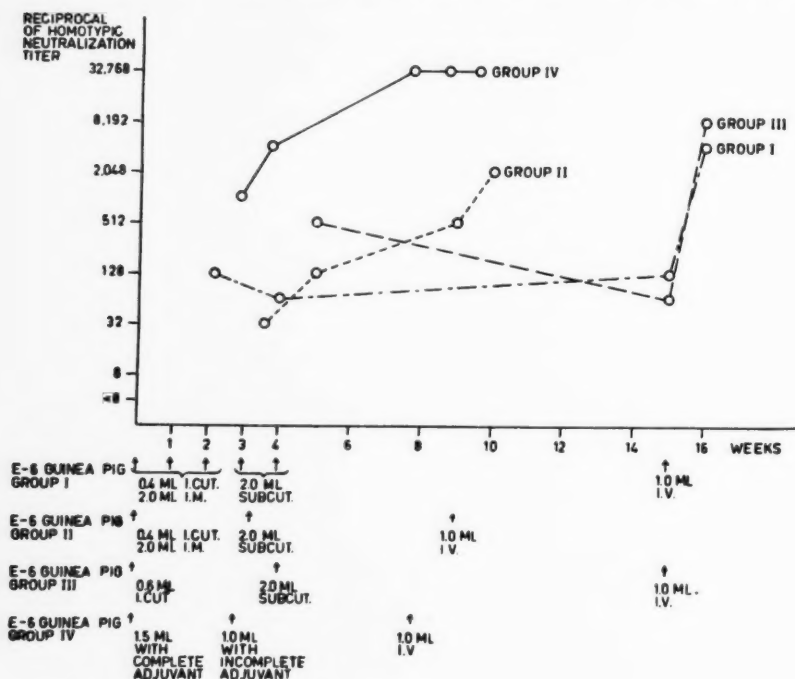


Fig. 2. — Development of homotypic neutralizing antibody titers in four groups of guinea pigs immunized with ECHO type 6 virus. Immunization schedules and routes indicated. Each point represents the titer of pooled sera of 2—4 guinea pigs.

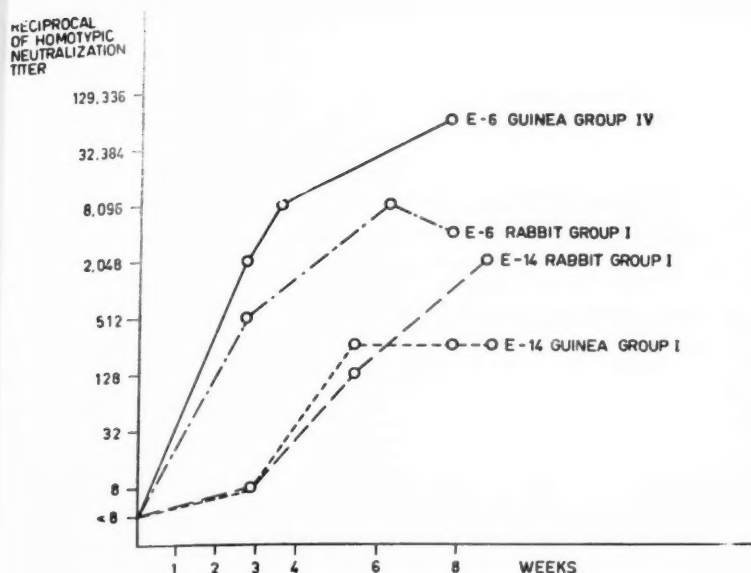


Fig. 3. — Homotypic neutralizing antibody titers in guinea pigs and rabbits immunized according to identical schedules with ECHO types 6 and 14 viruses. The schedule is indicated in Fig. 2. The immunizing doses for rabbits were four times larger than for guinea pigs.

A group of guinea pigs was immunized with concentrated ECHO type 4 virus according to the same schedule as was used in group IV, (see Fig. 2). One week after the third immunization the homotypic neutralizing antibody titer was 1:512 (Fig. 6).

*Rabbits.* — Homotypic neutralizing antibody titers in guinea pigs and rabbits immunized according to identical schedules with ECHO type 6 and 14 viruses is shown in Fig. 3. It will be noted that one week after the last immunization ECHO type 14 titers were higher in E-14 rabbit group I than in E-14 guinea pig group I, but ECHO type 6 titers were lower in rabbit group I than in guinea pig group IV.

*Effect of Freund's Adjuvants on the Homotypic Neutralizing Antibody Titers.* — Three groups of guinea pigs were immunized with three doses of ECHO type 14 virus. In the immunization of E-14 guinea pig group I, the first dose was mixed with Freund's complete adjuvant, and the second with incomplete adjuvant; in group II the first and second doses were mixed with incomplete

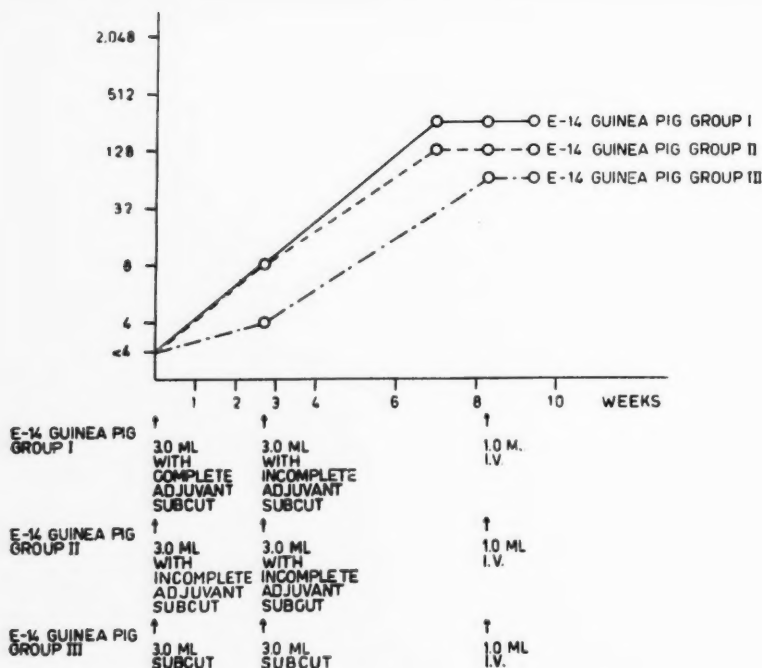


Fig. 4. — Development of neutralizing antibody titers in three groups of guinea pigs immunized with ECHO type 14 virus mixed with Freund's complete adjuvant, with Freund's incomplete adjuvant, and without adjuvant. Each point represents the titer of pooled sera of 6 guinea pigs.

adjuvant. No adjuvant was used in group III. The third dose in each group was given intravenously without adjuvant. The development of homotypic neutralizing antibody titers is shown in Fig. 4. One week after the last immunization the titers were highest in group I (1: 256) and lowest in group III (1: 64).

*Development of Non-specific, Heterotypic «Neutralization» Titers in Guinea Pigs and Rabbits.* — When the specificity of these ECHO antisera was tested, it was found that some of them clearly inhibited the cytopathogenic effect of some other ECHO types. This non-specific activity was correlated with the cytotoxic effect of these sera.

Development of homotypic neutralizing antibody titers and non-specific, heterotypic «neutralization» titers in guinea pigs immunized with ECHO type 6 and type 4 viruses is shown in Figs. 5 and 6. The non-specific activity was marked in ECHO type 4 antisera, where specific titers were only two or four times higher than non-

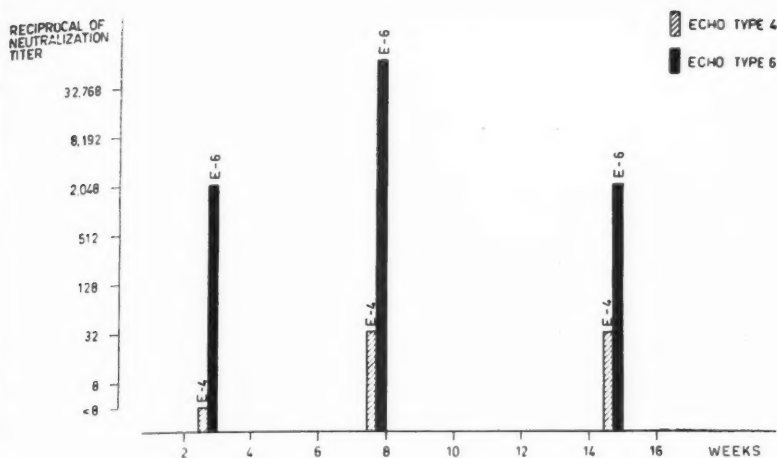


Fig. 5. — Development of homotypic neutralizing antibody titers and non-specific heterotypic (ECHO type 4) «neutralization» titers in E-6, guinea pig group IV. The immunization schedule and doses are indicated in Fig. 2.

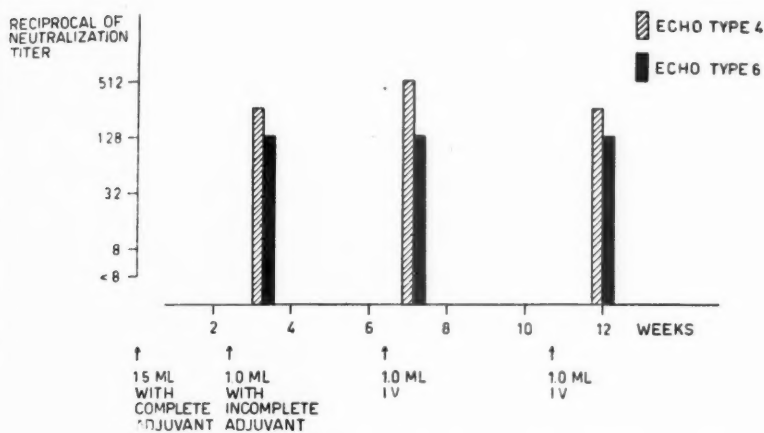


Fig. 6. — Development of homotypic neutralizing antibody titers and non-specific heterotypic (ECHO type 6) «neutralization» titers in E-4, guinea pig group I.

specific «neutralization» titers. The specific homotypic titers rose and fell quicker than the non-specific, heterotypic titers in guinea pigs immunized with ECHO type 6 virus, but by about the same rate in guinea pigs immunized with ECHO type 4 virus.

In order to find out the effect of adjuvants on the development of non-specific activity in guinea pig and rabbit sera, tests were

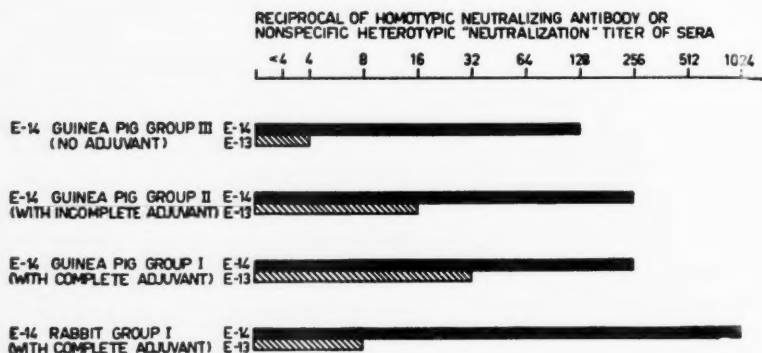


Fig. 7. — Homotypic neutralizing antibody titers and non-specific heterotypic (ECHO type 13) «neutralization» titer of three groups of guinea pigs and one group of rabbits immunized with ECHO type 14 virus mixed with Freund's complete adjuvant, with Freund's incomplete adjuvant, and without adjuvant. The immunization schedules and doses are indicated in Fig. 4. The sera were taken one week after the third immunization.

made on the homotypic and non-specific, heterotypic (ECHO type 13) activity of the pooled sera of E-14 guinea pig groups I, II, and III, and rabbit group I taken one week after the last immunization. It will be noted (Fig. 7) that adjuvants increased the non-specific heterotypic titers more than homotypic titers in guinea pigs. On the other hand, the proportion of homotypic and non-specific heterotypic titers was greater in rabbit group I (128) than in guinea pig group I (8).

*Effect of Adsorption of Guinea Pig Antisera with Normal Monkey Kidney Cells on Homotypic and Non-specific Heterotypic Neutralization Titers.* — A pooled ECHO type 4 antiserum taken one week after the third immunization of guinea pigs (immunization schedule in Fig. 6) was adsorbed once and twice with normal monkey kidney cells grown in cell culture. For the adsorption 20% of washed, packed cells were added to 1:4 dilution of antiserum; the suspension was incubated one hour at room temperature, and centrifuged 10 minutes at 2,000 r.p.m. An ECHO type 11 guinea pig antiserum prepared according to the same immunization schedule as ECHO type 4 antiserum was also adsorbed as indicated above. The adsorption decreased the non-specific heterotypic (ECHO type 6) «neutralization» titers 4 (adsorbed once) and 8 (adsorbed twice) times without decreasing the homotypic titers (Fig. 8).



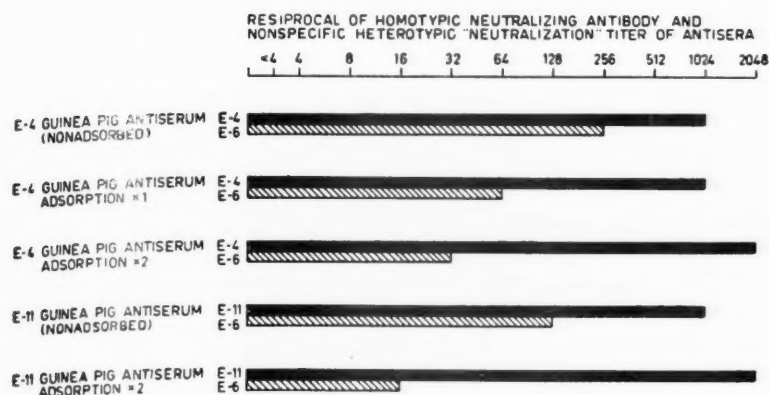


Fig. 8. — The effect of adsorption of ECHO type 4 and 11 guinea pig antisera with normal monkey kidney cells on homotypic neutralization antibody and non-specific heterotypic (ECHO type 6) "neutralization" titers.

TABLE 1

DEVELOPMENT OF HOMOTYPIC AND ANTI-HOST CF TITERS OF FOUR GROUPS OF GUINEA PIGS IMMUNIZED WITH ECHO TYPE 6 VIRUS. IN ADDITION TO THE IMMUNIZATION INDICATED IN FIGURE 2, THE ANIMALS WERE IMMUNIZED WITH 1.0 ML OF VIRUS GIVEN INTRAVENOUSLY AT 48 WEEKS

| Serum                          | Time after First Immunization | Reciprocal of Homotypic CF Titer | Reciprocal of Anti-host CF Titer |
|--------------------------------|-------------------------------|----------------------------------|----------------------------------|
| E-6, guinea pig group I ....   | 2 weeks                       | <8                               | <8                               |
| " " " " " " ....               | 4 "                           | 64                               | <8                               |
| " " " " " " ....               | 18 "                          | 128                              | 8                                |
| " " " " " " ....               | 48 "                          | 32                               | <8                               |
| " " " " " " ....               | 49 "                          | 32                               | <8                               |
| E-6, guinea pig group II ....  | 3 "                           | 8                                | <8                               |
| " " " " " " ....               | 5 "                           | 128                              | 32                               |
| " " " " " " ....               | 9 "                           | 256                              | 64                               |
| " " " " " " ....               | 48 "                          | 8                                | <8                               |
| " " " " " " ....               | 49 "                          | 16                               | <8                               |
| E-6, guinea pig group III .... | 5 "                           | 64                               | 64                               |
| " " " " " " ....               | 18 "                          | 512                              | 256                              |
| " " " " " " ....               | 48 "                          | 64                               | 8                                |
| E-6, guinea pig group IV ....  | 3 "                           | 32                               | 8                                |
| " " " " " " ....               | 9 "                           | 2048                             | 512                              |
| " " " " " " ....               | 48 "                          | 512                              | 64                               |
| " " " " " " ....               | 49 "                          | 2048                             | 512                              |

*Specific Virus and Anti-host CF Titers in Guinea-pig and Rabbit Antisera.* — The specific homotypic virus CF antibody titers in guinea pigs immunized with ECHO type 4 virus were higher than the anti-host CF titers during the early part of immunization, but they reached the same level (1:2048) two weeks after the third immunization. The CF activities in antisera of four ECHO type 6 guinea pig groups are shown in table 1. Virus CF titers in each group correlated well with the homotypic neutralization titers shown in Fig. 2. The proportion of homotypic virus CF titer to anti-host CF titer was about the same except in group I where, however, the specific virus CF titers were lowest. The drop of both titers was fairly proportional in each group as well as the rise of the titers one week after the booster at 48 weeks.

The homotypic virus CF titers of rabbit antisera were 2 to 8 times lower than corresponding guinea pig antisera, *e.g.* one week after the third immunization E-14 rabbit group I had a CF titer of 1:256, whereas E-14 guinea pig group I had a CF titer of 1:512. In E-6 rabbit group I and E-6 guinea pig group IV the homotypic CF titers were 1:256 and 1:2048. Most of the rabbit antisera tested were anticomplementary up to dilutions of 1:16 and 1:32 even when inactivated 30 minutes at 60°C.

TABLE 2

RECIPROCAL OF HOMOTYPIC NEUTRALIZING ANTIBODY TITERS AND HETEROTYPIC NONSPECIFIC «NEUTRALIZATION» TITERS OF ECHO TYPES 4, 5, 6, 11, 12, 13, AND 14 GUINEA PIG ANTISERA. 0 = 32; ++++ = COMPLETE DEGENERATION OF CELLS; +++, ++, + = DIFFERENT DEGREES OF DEGENERATION; — = NO DEGENERATION

| Virus                                  | ECHO Guinea Pig Sera |      |       |      |      |      |      |
|--|----------------------|------|-------|------|------|------|------|
|  | E-4                  | E-5  | E-6   | E-11 | E-12 | E-13 | E-14 |
| E-4                                    | 512                  | 64   | 0     | 64   | 32   | 32   | 0    |
| E-5                                    | 64                   | 8192 | 0     | 128  | 32   | 64   | 0    |
| E-6                                    | 128                  | 128  | 32768 | 128  | 64   | 32   | 0    |
| E-11                                   | 0                    | 32   | 0     | 8192 | 0    | 0    | 0    |
| E-12                                   | 0                    | 0    | 0     | 0    | 2048 | 0    | 0    |
| E-13                                   | 128                  | 128  | 0     | 64   | 32   | 512  | 0    |
| E-14                                   | 64                   | 128  | 0     | 64   | 64   | 64   | 2048 |
| Cytotoxic activity in dilution 1:16 .. | +++                  | +++  | —     | ++++ | ++   | +++  | —    |

*Homotypic and Heterotypic Neutralization and CF Titers of ECHO Type 4, 5, 6, 11, 12, 13, and 14 Guinea-pig Antisera.* — Homotypic neutralizing antibody titers and heterotypic, non-specific «neutralization» titers of ECHO type 4, 5, 6, 11, 12, 13, and 14 guinea pig antisera are shown in table II, where the cytotoxic activity in serum dilution 1: 16 is also indicated. All antisera were prepared by immunizing the animals according to the same schedule as was used for ECHO type 6 guinea pig group IV, i.e. three injections, 3 and 7 weeks apart, the first with Freund's complete adjuvant, the second with Freund's incomplete adjuvant, and the third given intracardially without adjuvant, and bleeding one week after the last injection. It will be noted that in the antisera with no cytotoxic activity in a dilution of 1: 16 no heterotypic «neutralization» could be obtained in a dilution of 1: 32. That the heterotypic reactions were non-specific could be seen from the specificity of these same antisera in the CF test (table 3).

TABLE 3

RECIPROCAL OF HOMOTYPIC AND HETEROTYPIC CF TITERS OF ECHO TYPES 4, 5, 6, 11, 12, 13, and 14 Guinea Pig Sera. 0 = <8

| Fluorocarbon-treated ECHO CF Antigens | ECHO Guinea Pig Sera |      |      |      |      |      |      |
|---------------------------------------|----------------------|------|------|------|------|------|------|
|                                       | E-4                  | E-5  | E-6  | E-11 | E-12 | E-13 | E-14 |
| E-4                                   | 1024                 | 0    | 0    | 0    | 0    | 0    | 0    |
| E-5                                   | 0                    | 4096 | 0    | 0    | 0    | 0    | 0    |
| E-6                                   | 0                    | 0    | 4096 | 0    | 0    | 0    | 0    |
| E-11                                  | 0                    | 0    | 0    | 2048 | 0    | 0    | 0    |
| E-12                                  | 0                    | 0    | 0    | 0    | 128  | 0    | 0    |
| E-13                                  | 0                    | 0    | 0    | 0    | 0    | 1024 | 0    |
| E-14                                  | 0                    | 0    | 0    | 0    | 0    | 0    | 512  |

## DISCUSSION

A common method of preparing virus antisera has been to immunize the animals one or more times weekly during several successive weeks. Usually, the more difficult the preparation of antiserum to a certain virus, the greater the number of immunizing injections given. The data presented indicate that ECHO virus antisera with high neutralization and CF titers could be

prepared in guinea pigs or rabbits with two or three injections of virus at proper intervals. Repeated weekly immunization of animals did not increase the neutralization titers, — on the contrary the titers were considerably higher in animals immunized with only two or three injections.

Although Freund's adjuvants clearly increased the homotypic ECHO neutralizing antibody titer in guinea pigs, they increased the non-specific, heterotypic titer even more (Figs. 4 and 7). It is true that the experiments with Freund's adjuvants were made with concentrated and sonic treated antigens including large amounts of host antigens. Hence, with purer virus antigens the effect of the adjuvants might be more specific. Their effect might also depend on the animals used for the preparation of antisera, since the rabbits immunized with virus mixed with adjuvants showed less non-specific activity than the guinea pigs (Fig. 7).

As regards the choice of laboratory animal for the preparation of ECHO virus antisera, monkeys have the great advantage of producing only a low titer of host antibodies, when immunization antigens consist of monkey kidney cell culture grown virus (6). On the other hand, monkeys may contract a natural infection with related viruses causing cross-reactions in antisera and making necessary extensive cross-neutralization tests of pre- and post-immunization samples of monkey sera. In addition, monkeys are not always available. Hence, rabbits and guinea pigs are the animals which have mostly been used in the preparation of virus antisera for small scale use. In this study rabbits produced higher ECHO type 14 and lower ECHO type 6 homotypic neutralization titers than guinea pigs, but ECHO type 14 rabbit antiserum, which was the only single rabbit serum in cross-reaction tests, gave lower heterotypic reactions. This may indicate that the rabbit is preferable for the preparation of ECHO antisera to be used in neutralization tests. Rabbit sera, however, are often anticomplementary in low dilutions, whereas guinea pig sera could be regularly used even undiluted in the CF test, and, moreover, the specific ECHO virus CF antibody titers may be higher in guinea pigs than in rabbits.

The great strength of the non-specific reactions of ECHO guinea pig antisera obtained in this study could partly be explained by the fact that many of the antigens used were prepared by sonic vibration and ultracentrifugation, which may be more favourable

for concentration of host than of virus antigens. However, it cannot entirely explain the cross-reactions obtained, since in the latter part of immunization with an unconcentrated ECHO antigen a clear non-specific, heterotypic cross-reaction could also be obtained (Fig. 5).

It is interesting to note that none of the experimental animals showed «real» cross-reactions between ECHO viruses, although serological studies of human infections suggest that there may be antigens shared by certain ECHO viruses.

#### SUMMARY

Groups of mice, guinea pigs and rabbits were immunized according to various schedules with ECHO viruses grown in monkey kidney cell culture. The course of development of homotypic neutralization titers in sera of mice, and of homotypic neutralizing and CF antibody titers, non-specific, heterotypic «neutralization» titers, cytotoxicity and host CF activity in sera of guinea pigs and rabbits was studied.

The homotypic neutralization titers of mouse antisera were 1:512 or less. In guinea pig antisera the homotypic neutralization titers were up to 1:32,768 and in rabbits in the same range, if the animals were immunized with two or three injections of virus at intervals of 3 and 4 weeks. After repeated weekly immunization the homotypic neutralization titers were lower. The non-specific heterotypic «neutralization» titers, which were correlated with the cytotoxic activity of the antisera, were considerable, especially in guinea pigs immunized with concentrated ECHO virus antigens. In guinea pigs Freund's adjuvants increased the homotypic neutralizing antibody titers but even more the non-specific, heterotypic titers. The specific virus CF antibody titers in guinea pig antisera were correlated with the homotypic neutralization titers. The host CF titers were high, in some guinea pig antisera as high as the specific virus CF titer.

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## AFFINITY OF CERTAIN SURFACE-ACTIVE SUBSTANCES TO C-REACTIVE PROTEIN

### PART I

by

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In previous studies (1, 2, 3) it was shown that certain surface active substances produce when using a given technic, a flocculation reaction in sera containing C-reactive protein. The first studies were made with the following products: Span 60, which gave the best results, Tween 61 and Span 65, which yielded weaker yet definite reactions, Span 80 and Brij 30, giving only weak reactions, and Tween 85, G-2859 and G-1425, with which no reaction was obtained. In the present work (Part I) an attempt has been made to determine more closely the component or components in the above mentioned and certain other products on which this reaction is based.

*Span 60.* — According to the manufacturer's statement (4), Span 60 is a complicated mixture of fatty acid esters of sorbitol anhydrides, chiefly sorbitan monostearate. We performed at first a gradual precipitation from ethanol using temperature intervals of 5°C and 10°C (method I), a starting solution of 10 gr of Span 60/100 ml of ethanol, and a precipitation time of 2 hrs. at each interval. From each precipitate an «antigen» containing 0.25 per cent of the fraction, 0.3 per cent of lecithin, and 0.9 per cent of

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<sup>1</sup> Aided by a grant from the Sigrid Jusélius Foundation.

cholesterol in ethanol was prepared. A known serum containing C-reactive protein was tested with this antigen and a parallel test was made with the original Span 60 antigen (APC test). The reaction was carried out with twofold serum dilutions. The results are presented in Table 1, which also shows the saponification values of the fractions.

TABLE 1  
ACTIVITIES OF FRACTIONS OF SPAN 60 OBTAINED BY GRADUAL PRECIPITATION

| Fraction   | Activity |     |     |     |      |      |      | S.V. |
|------------|----------|-----|-----|-----|------|------|------|------|
|            | 1/1      | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 |      |
| > +20°C    | —?       | ±   | +   | +   | —?   | —    | —    | 175  |
| +20°—+10°C | +++      | +++ | +++ | +++ | —?   | —    | —    | 168  |
| +10°—± 0°C | +++      | +++ | +++ | ++  | ±    | —    | —    | 155  |
| ± 0°—-15°C | +        | ++  | ++  | ++  | ±    | —?   | —    | 150  |
| < -15°C    | —        | —?  | ±   | ±   | —?   | —?   | —    | 110  |
| APC .....  | +++      | +++ | +++ | ±   | ±    | —    | —    |      |

In the above described fractionations about 20–30 per cent of the substance was usually precipitated above +20°C and the amount of fraction remaining in the solution below -15°C was on the average 10–15 per cent. The fractions over +20°C were

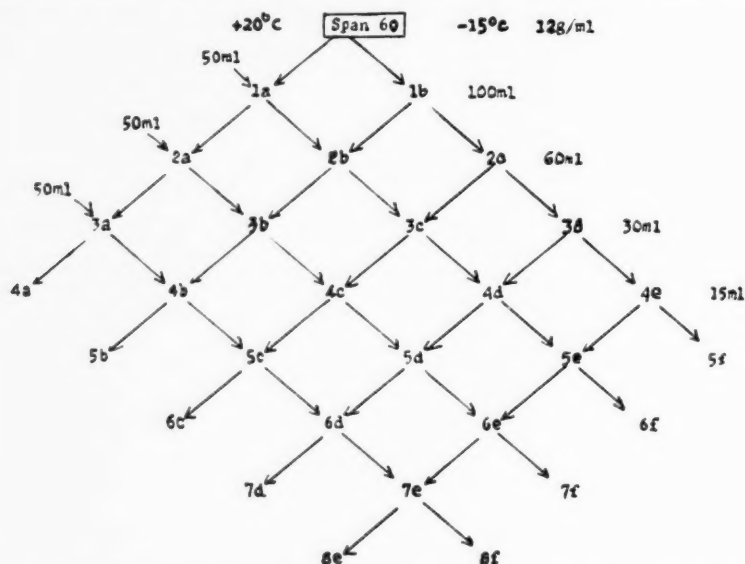


Fig. 1. — Scheme of fractional crystallization of the active fraction of Span 60.



white and powdery, the lower fractions waxy, and those under  $-15^{\circ}\text{C}$  semisolid only.

Proceeding further with the experiments we carried out a fractional crystallisation, method II, of the middle fraction of Span 60 in the range  $+20^{\circ}\text{C}$  to  $-15^{\circ}\text{C}$  from ethanol, as shown in Fig. 1.

The activity of the fractions obtained in fractional crystallisation and the saponification values of some of the fractions are presented in Table 2.

TABLE 2  
ACTIVITIES OF FRACTIONS OBTAINED BY FURTHER FRACTIONAL CRYSTALLIZATION  
OF THE MIDDLE FRACTION OF SPAN 60

| Fraction | Activity |     |     |     |      |      |      | S.V. |
|----------|----------|-----|-----|-----|------|------|------|------|
|          | 1/1      | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 |      |
| 4a       | —?       | ±   | ±   | —?  | —?   | —    | —    |      |
| 5b       | —        | —?  | —?  | —?  | —    | —    | —    |      |
| 6c       | ±        | +   | +   | ±   | —?   | —    | —    |      |
| 7d       | —?       | —?  | —?  | —?  | —    | —    | —    |      |
| 8e       | —?       | +   | +   | +   | —?   | —    | —    |      |
| 8f       | +++      | +++ | +++ | +++ | +    | —    | —    | 169  |
| 7f       | ++       | +++ | ++  | ++  | ±    | —    | —    | 169  |
| 6f       | ++       | +++ | +++ | ++  | —?   | —    | —    | 161  |
| 5f       | ++       | +++ | +++ | +++ | ++   | ±    | —    | 152  |
| APC      | +++      | +++ | +++ | ±   | ±    | —    | —    |      |

The activity remained in the mother liquors and the closeness of the saponification values of the active fractions is striking. The theoretical saponification values of sorbitan stearates are as follows:

|          |              |                |       |     |
|----------|--------------|----------------|-------|-----|
| Sorbitan | monostearate | Saponif. value | ..... | 130 |
| »        | di           | »              | »     | 160 |
| »        | tri          | »              | »     | 172 |
| »        | tetra        | »              | »     | 180 |

On comparison of the saponification values of the active fractions with the theoretical values it appears probable that the active fractions most closely correspond to mono- and distearates.

*Span 65.* — According to the manufacturer's statement this is closest to sorbitan tetrastearate. The saponification value of

Span 65, which is 180, corresponds very accurately to the theoretical saponification value of sorbitan tetrastearate. Fractionation was carried out as above, method I. The substance, however, was not totally dissolved in boiling alcohol. The results are listed in Table 3.

TABLE 3  
ACTIVITIES OF FRACTIONS OF SPAN 65 OBTAINED BY GRADUAL PRECIPITATION

| Fraction   | Activity |     |     |     |      |      |      | S.V. |
|------------|----------|-----|-----|-----|------|------|------|------|
|            | 1/1      | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 |      |
| > +20°C    | —        | —   | —   | —   | —    | —    | —    | 187  |
| +20°—+10°C | —?       | ±   | +   | ±   | —?   | —    | —    | 176  |
| +10°—± 0°C | +        | ++  | +++ | ++  | +    | —    | —    | 168  |
| ± 0°—-15°C | +++      | +++ | +++ | +++ | ±    | —    | —    | 144  |
| < -15°C    | +        | ++  | ++  | +   | —?   | —    | —    | 131  |
| APC .....  | +++      | +++ | +++ | ±   | ±    | —    | —    |      |

The saponification values of the active fractions of Span 60 and Span 65 were in the range 137–169, the average being 157. Compared with the theoretic values the range of activity extended from 1.1-stearate to 2.55-stearate, the average degree of substitution being 1.8. The effect on the serologic reaction of the less and more esterified components will be dealt with in a later stage of the present work (Part III).

*Tween 65.* — This is chiefly poly-(oxyethylene)-20-sorbitan tristearate according to the manufacturer. Its theoretical saponification value would be 90. Table 4 shows the results of fractionation, method I.

TABLE 4  
ACTIVITIES OF FRACTIONS OF TWEEN 65 OBTAINED BY GRADUAL PRECIPITATION

| Fraction    | Activity |     |     |     |      |      |      | S.V. |
|-------------|----------|-----|-----|-----|------|------|------|------|
|             | 1/1      | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 |      |
| > +10°C ..  | —        | —   | —   | —   | —    | —    | —    | 105  |
| < +10°C ..  | +        | ±   | ±   | —?  | —?   | —?   | —    | 90   |
| Tween 65 .. | —?       | —?  | —?  | —?  | —    | —    | —    | 93   |
| APC .....   | +++      | +++ | +++ | +++ | +++  | +    | —    |      |

The saponification value of the active fraction appears to be closest to that of tristearate.

*Span 20.* — According to the manufacturer's statement this is closest to sorbitan monolaurate. Since the product is almost syrupy at room temperature, no fractional precipitation was performed. The activity of the product compared with that of the original Span 60 was as follows:

|            | 1/1 | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 | S.V. |
|------------|-----|-----|-----|-----|------|------|------|------|
| Span 20 .. | —?  | ±   | +   | +   | +    | +    | —?   | 162  |
| APC .....  | ++  | +++ | +++ | +++ | +++  | +    | —    |      |

*Span 40.* — According to the manufacturer this product is closest to sorbitan monopalmitate. The theoretical saponification values of sorbitan palmitates are as follows:

|          |               |      |     |
|----------|---------------|------|-----|
| Sorbitan | monopalmitate | S.V. | 134 |
| »        | di            | »    | 167 |
| »        | tri           | »    | 180 |
| »        | tetra         | »    | 191 |

The results of fractional precipitation, method I, are given in Table 5.

TABLE 5  
ACTIVITIES OF FRACTIONS OF SPAN 40 OBTAINED BY GRADUAL PRECIPITATION

| Fraction      | Activity |     |     |      |      |      |      | S.V. |
|---------------|----------|-----|-----|------|------|------|------|------|
|               | 1/1      | 1/2 | 1/4 | 1/8  | 1/16 | 1/32 | 1/64 |      |
| > +20°C ..    | +        | ++  | +++ | ++++ | ++   | +    | —    | 173  |
| +20°—+ 5°C .. | ++       | +++ | +++ | ++++ | +++  | +    | —    | 149  |
| 5°—-10°C ..   | +++      | +++ | +++ | +++  | ++   | +    | —    | 146  |
| < -10°C ..    | —        | —?  | —?  | ±    | ±    | ±    | —?   | 107  |
| Span 40 ..... | +        | ++  | +++ | ++++ | +++  | —?   | —    | 140  |
| APC .....     | ++       | +++ | +++ | +++  | +++  | +    | —    |      |

Compared with the theoretical values the serologically most active fraction are between mono- and dipalmitate.

The observations presented above indicate that the affinity of fatty acid esters of sorbitan and polyoxyethylene sorbitan to C-reactive protein probably is not a property of any single chemical substance dealt with above, but rather that compounds derived of different fatty acids and sorbitan and polyoxyethylene sorbitan have in general an affinity to C-reactive protein. Since it appeared difficult to get more precise facts on the nature of the reactive

substances by analytic methods, we undertook the synthetisation of fatty acid esters of polyols, results of which are presented in Part II and III of this paper.

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## AFFINITY OF CERTAIN SURFACE-ACTIVE SUBSTANCES TO C-REACTIVE PROTEIN

### PART II

by

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(Received for publication November 8, 1960)

This part of the present paper deals with the affinity of certain polyol fatty acid esters, synthesized by us, to C-reactive protein. The affinity was determined in the same manner as in Part I by carrying out a flocculation reaction in different dilutions of a known serum containing C-reactive protein, using as «antigen» the substance under examination with added lecithin and cholesterol (1, 2, 3, 4).

*Isosorbide Stearate.* — The isosorbide was prepared according to Hockett *et al.* (5) and esterified with an acid catalyst without any solvent, and the water formed was removed azeotropically with toluene. The catalyst was p-toluene sulfonic acid. The synthesized ester was fractionated from ethanol using the technic described in Part I (method I), the catalyst being soluble in alcohol remained in fractions below  $-15^{\circ}\text{C}$ . Removal of the catalyst was not considered necessary, since these fractions were generally inactive.

The activity of the fractions obtained at different temperatures is presented in Table 1, which also shows the saline control of the antigen, the negative serum control, and the saponification values and molecular weights of some fractions.

<sup>1</sup> Aided by a grant from the Sigrid Jusélius Foundation.

TABLE 1  
ACTIVITIES OF DIFFERENT FRACTIONS OF ISORBIDE STEARATE SYNTHESIS

| No.   | Fraction  | Activity |     |     |     |      |      |      |       | Neg.Ser. | NaCl | S.V. | M.W.    |
|-------|-----------|----------|-----|-----|-----|------|------|------|-------|----------|------|------|---------|
|       |           | 1/1      | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 | 1/128 |          |      |      |         |
| 50    | > +30°    | —        | —   | —   | —   | —    | —    | —    | —     | —        | +    | 161  | 640—700 |
| 51    | +30°—+10° | —        | —   | —   | —   | —    | —    | —    | —     | —        | +    | 136  | 410—470 |
| 52    | +10°—15°  | +        | +   | +   | +   | +    | +    | —?   | —     | —        | —    | —    | —       |
| 53    | <—15°     | +        | +   | +   | +   | +    | ±    | —    | —     | —        | —    | —    | —       |
| 50—53 |           | —        | —   | —   | —   | —    | —    | —    | —     | —        | +    | —    | —       |
| APC   |           | +        | +   | +   | +   | ±    | —    | —    | —     | —        | —    | —    | —       |

TABLE 2  
ACTIVITIES OF DIFFERENT FRACTIONS OF ARLITAN STEARATE SYNTHESIS

| No.     | Fraction     | Activity |     |     |     |      |      |      |       | Neg.Ser. | NaCl | S.V. |
|---------|--------------|----------|-----|-----|-----|------|------|------|-------|----------|------|------|
|         |              | 1/1      | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 | 1/128 |          |      |      |
| 68      | All fr. tog. | —        | —?  | —   | —?  | —    | —    | —    | —     | —        | —    | 142  |
| 69      | > +30°       | —?       | —   | —   | —   | —    | —    | —    | —     | —        | +    | 161  |
| 70      | +30°—+10°    | —        | —   | —   | —   | —    | —    | —    | —     | —        | +    | 157  |
| 71      | +10°—0°      | +        | +   | +   | +   | ±    | —?   | —?   | —?    | —?       | —?   | 144  |
| 72      | ± 0°—15°     | +        | +   | +   | +   | —?   | —?   | —?   | —?    | —?       | —?   | 132  |
| 73      | <—15°        | +        | +   | +   | +   | ±    | —?   | —?   | —?    | —?       | —?   | 120  |
| Arlitan |              | —        | —   | —   | —   | —    | —    | —    | —     | —        | —    |      |
| APC     |              | +        | +   | +   | +   | ±    | —    | —    | —     | —        | —    |      |

It was possible to isolate fraction No. 50 very accurately from the other fractions. Its precipitation began at ca. 55°C, when flocculent crystals were formed, M.P. +77.5°C. The saponification value of the fraction, which is 161, lies very close to the theoretical saponification value of isosorbide distearate, which is 165. The fraction was completely inactive. A combination of the fractions numbered 50–53 in the same proportions as they occurred in the synthesis mixture also gave a negative reaction. We shall revert to this point in Part III. In a control test the starting substances in the synthesis, stearic acid and isosorbide, gave negative results. The active fraction +10°C — —15°C, on basis of its saponification value, corresponded quite closely to isosorbide monostearate, which has a saponification value of 136. Monostearate may be either a carbon-2 or a carbon-5 ester; this point was not given further study.

*Arlitan Stearate.* — Arlitan, or 1,4-sorbitan, which was used as the starting material, was prepared according to Soltzberg *et al.* (6). Owing to the conditions of esterification, polyol anhydration occasionally occurred in this synthesis, as also did in the following two syntheses. The synthesized product was fractionated from ethanol and the activity of the fractions is shown in Table 2.

*Sorbitol Stearate.* — Synthesis was carried out as above. Table 3 shows the activity of the fractions.

On examination of the results of the three syntheses we observe that the results possible are slightly better with increasing OH content of the alcohol component. However, differences are not very marked.

*Sorbitol Palmitate.* — To shed light on the specificity of the fatty acid, the synthesis was carried out with palmitic acid. The results are given in Table 4.

This relatively little shortening of the fatty acid chain had no appreciable effect on the reaction. In fact, the reaction seemed to be clearer when using palmitate rather than stearate.

*Mannitol Stearate.* — To bring out the effect of the polyol configuration, mannitol stearate was synthesized and the mixture was fractionated in the same manner as previously. The results are shown in Table 5.

The activity of mannitol stearate is thus fully equivalent to the corresponding sorbitol compound.

TABLE 3  
ACTIVITIES OF DIFFERENT FRACTIONS OF SORBITOL STEARATE SYNTHESIS

| No.      | Fraction     | Activity |     |     |     |      |      |      | Neg. Ser. | NaCl | S.V. |
|----------|--------------|----------|-----|-----|-----|------|------|------|-----------|------|------|
|          |              | 1/1      | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 |           |      |      |
| 79       | All fr. tog. | —?       | +   | +   | +   | —?   | —    | —    | —         | —?   | 141  |
| 74       | > +30°       | —        | —   | —   | —   | —    | —    | —    | —         | +    | 175  |
| 75       | +30°—+15°    | —        | —   | —   | —   | —    | —?   | —    | —         | +    | 164  |
| 76       | +15°—0°      | ++       | ++  | ++  | ++  | ±    | —    | —    | —         | ±    | 148  |
| 77       | 0°—15°       | ++       | ++  | ++  | ++  | ±    | —    | —    | —         | —    | 144  |
| 78       | <—15°        | —?       | +   | +   | +   | +    | —?   | —    | —         | —?   | 106  |
| Sorbitol |              | —        | —   | —   | —   | —    | —    | —    | —         | —    |      |
| APC      |              | ++       | ++  | ++  | ++  | ±    | —    | —    | —         | —    |      |

TABLE 4  
ACTIVITIES OF DIFFERENT FRACTIONS OF SORBITOL PALMITATE SYNTHESIS

| No. | Fraction     | Activity |     |     |     |      |      |      | Neg. Ser. | NaCl | S.V. |
|-----|--------------|----------|-----|-----|-----|------|------|------|-----------|------|------|
|     |              | 1/1      | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 |           |      |      |
| 80  | All fr. tog. | ++       | ++  | ++  | ++  | —    | —    | —    | —         | —    | 145  |
| 81  | > +30°       | —        | —   | —   | —   | —    | —    | —    | —         | +    | 173  |
| 82  | +30°—+15°    | +        | +   | +   | +   | —?   | —    | —    | —         | +    | 172  |
| 83  | +15°—0°      | ++       | ++  | ++  | ++  | —    | —    | —    | —         | —    | 158  |
| 84  | 0°—15°       | ++       | ++  | ++  | ++  | +    | —    | —    | —         | —    | 148  |
| 85  | <—15°        | —        | —   | —   | —   | —?   | —?   | —    | —         | —    | 117  |
| APC |              | ++       | ++  | ++  | ++  | ±    | —    | —    | —         | —    |      |



The above mentioned »dry syntheses» had the drawbacks that mono-, di-, tri-, etc. esters were produced, there was definite anhydration of the alcohol component under the experimental conditions, and esterification evidently occurred with equal ease at carbon 1 as at carbon 6. For this reason we repeated the esterification in a solution using dimethyl formamide as solvent, according to Osipow *et al.* (7). The alcohol component and the methyl or ethyl ester of the fatty acid were dissolved in the solvent. The catalyst was potassium carbonate. Under these conditions it was to be expected that when given amounts of original substances are used, conversion will take place in the solution in the manner that the end results will be, for example, chiefly monoesters. York *et al.* (8), among others, have demonstrated that upon esterification of, for instance, saccharose the monosubstitution is directed mostly upon carbon 6, which is to be expected on the basis of the generally greater activity of the primary alcohol groups. Using this »solution method» we repeated the syntheses of isosorbide stearate, arlitan stearate and sorbitol stearate. The synthesized mixture was washed in boiling water and the undissolved material was dissolved in ethanol and subjected to fractionation. The activity determinations and saponification values of the different fractions showed that the results obtained in the solution syntheses of sorbitol stearate and arlitan stearate corresponded fairly closely to the earlier results, with the exception that these syntheses yielded almost exclusively monoesters and that their activity was high. On the other hand, in the synthesis of isosorbide ester, even when the solution method was used, two fractions were obtained, one corresponding to the monoester and the other to the diester. The first mentioned was highly active, but the latter was not only inactive but it inhibited the activity of the first mentioned. The result thus was similar to that obtained in dry synthesis. This result is comprehensible when we consider the structure of isosorbide, which contains only two secondary OH groups that may be presumed to have approximately equal activity: the result is mono- and diesters. Sorbitol and arlitan, again which contain primary alcohol groups, yielded only monoesters. This may be regarded as indirect evidence that esterification in the solution method is selectively directed to the primary alcohol groups. On the basis of the positive results obtained with isosorbide, on the other

TABLE 5  
ACTIVITIES OF DIFFERENT FRACTIONS OF MANNITOL STEARATE SYNTHESIS

| No.             | Fraction     | Activity |     |     |     |      |      |      |       | Neg. Ser. | NaCl | S.V. |
|-----------------|--------------|----------|-----|-----|-----|------|------|------|-------|-----------|------|------|
|                 |              | 1/1      | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 | 1/128 |           |      |      |
| 86              | All fr. tog. | ±        | +   | +   | +   | ±    | —?   | —    | —?    | —         | —    | 142  |
| 87              | > +30°       | —        | —   | —   | —   | —    | —    | —    | —     | —         | ±    | 169  |
| 88              | +30°—+20°    | +        | +   | +   | +   | +    | ±    | —    | —     | —         | —    | 158  |
| 89              | +20°—+5°     | ±        | +   | +   | +   | ±    | —?   | —    | —     | —         | +    | 153  |
| 90              | +5°—15°      | +        | +   | +   | +   | +    | +    | —?   | —?    | —         | —    | 140  |
| 91              | < —15°       | —        | ±   | +   | +   | +    | +    | —    | —?    | —         | —    | 97   |
| Mannitol<br>APC |              | +        | +   | +   | +   | ±    | —?   | —    | —?    | —         | —    | 000  |

TABLE 6  
EFFECT OF METHYLATION ON ACTIVITY

| Fraction        | Activity |     |     |     |      |      |      | Neg. Ser. | NaCl | S.V. |
|-----------------|----------|-----|-----|-----|------|------|------|-----------|------|------|
|                 | 1/1      | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 |           |      |      |
| 80              | ++       | ++  | ++  | ++  | —    | —    | —    | —         | —    | 145  |
| Methyl. n:o 80  | —        | —?  | ±   | ±   | ±    | —?   | —    | —         | —    | 144  |
| 128             | ±        | +   | +   | +   | +    | —?   | —    | —         | —    | 152  |
| Methyl. n:o 128 | —        | —?  | —?  | —?  | —?   | —?   | —    | —         | —    | 155  |
| APC             | ++       | ++  | ++  | ++  | +    | —    | —    | —         | —    |      |

hand, it may be presumed that the esterification need not take place in the primary alcohol group in order that the ester produced would have affinity to C-reactive protein. It is therefore probable that the position of the fatty acid chain does not play a decisive rôle in the activity.

*Disaccharide Stearates.* — Using the solution method, saccharose and trehalose stearates were synthesized. Both preparations of synthesis showed definite activity against C-reactive protein. The active fractions were closest to distearates.

*Methylation.* — Since isosorbide distearates showed most clearly that the activity was lost when all OH groups were esterified, we considered it to be of interest to find out whether free hydroxyl groups were indispensable for binding to C-reactive protein. Two samples were therefore methylated according to Purdie *et al.* (9). One of the samples was fraction N:o 80, which has been presented above and which was a mixture resulting from the sorbitol palmitate synthesis. The other sample was fraction  $+5^{\circ}\text{C} - -15^{\circ}\text{C}$  obtained in the solution synthesis of isosorbide stearate. The results are shown in Table 6.

It is observed from the results that the activity was almost completely lost. It is known that methylation of the last hydroxyl group of polyol usually is difficult and requires, among others, a large excess of methylating agents. It may be possible that the methylated products still contain small amounts of free OH groups that give rise to slightly positive reactions. It is therefore probable that the presence of free OH groups in the esters is a prerequisite for the activity exhibited by substances of the kind now studied towards C-reactive protein.

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## AFFINITY OF CERTAIN SURFACE-ACTIVE SUBSTANCES TO C-REACTIVE PROTEIN

### PART III

by

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This part of our study deals with the effect of some variables in the APC-test and in its analogues. At the end of this paper a summary is given of the observations in the different parts of the present work (8, 9).

The flocculation technique of the APC-test and its analogues is similar to the VDRL test used in syphilis serology (1) and is based on the addition of a given amount of the substance under examination to a solution of absolute ethanol containing 0.9 per cent cholesterol and 0.3 per cent lecithin, after which the alcoholic solution is precipitated by pipetting it slowly into a saline or buffer solution. Needle-shaped complex crystals are thus produced. When this »antigen» is added to serum, a precipitate is formed consisting of antigen crystals and the reactive component of the serum. When cardiolipin is used as the added substance, the test is called the VDRL test, and when sitolipin is used, it is the sitolipin test (2). Analogously, an alcoholic extract of the Tb-bacillus may be used, enabling the examination of the presence of Tb reagents in the serum (3, 4). Using Span 60 as the added substance the test may

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be used for measurement of the C-reactive protein in the serum, and the test is termed the APC test (5, 6, 7). All of these added substances have the common characteristic that they are or contain long chain fatty acids, which in most cases are bound to polyols by ester linkages. At least a part of the added substances, which react in the test with C-reactive protein, also react without the addition of cholesterol and lecithin but the reactions are weak (4). The hydrophilic character of the antigen crystals has a very significant influence on the reaction. If the crystals are hydrophobic, precipitation may occur even in saline solution. When the amount of cholesterol is kept constant, the optimum amount of lecithin is determined by the fact that the reactivity of the complex crystals usually increases with increasing amounts of lecithin, which raises the hydrophilic character of the complex until a point is reached where the formation of macroscopic crystals is inhibited and an opalescent antigen solution is obtained. At this point the reaction sensitivity falls abruptly. The third substance used in the crystals also influences this optimum ratio. For example, sitolipin acts to some extent like lecithin and increases the solubility; some other substances may possibly reduce it. The following table shows the effect of various amounts of lecithin on the results of the reaction when 0.9 per cent of cholesterol, 0.3 per cent of Span 60 and varying amounts of lecithin are used.

TABLE 1

EFFECT OF THE VARIATION OF LECITHIN CONCENTRATION IN THE APC ANTIGEN

| Per Cent of<br>Lecithin | Activity |     |     |     |      |      | Neg.Ser. | NaCl |
|-------------------------|----------|-----|-----|-----|------|------|----------|------|
|                         | 1/1      | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 |          |      |
| 0                       | ±        | ±   | ±   | ±   | ±    | ±    | ±        | ±    |
| 0.15                    | ±        | +   | +   | ±   | —    | —    | —        | —    |
| 0.30                    | ++       | ++  | ++  | +   | ±    | —    | —        | —    |
| 0.45                    | ±        | ±   | ±   | —   | —    | —    | —        | —    |

Using varying concentrations of Span 60, constant concentration of cholesterol and three different concentrations of lecithin, the results shown in Table 2 are obtained.

As was seen in the tables in the previous parts of the present work, the reaction of very many substances may be inhibited when the test is carried out with undiluted or slightly diluted

TABLE 2

EFFECT OF THE VARIATION OF LECITHIN AND SPAN 60 CONCENTRATION IN THE APC ANTIGEN. CHOLESTEROL CONCENTRATION CONSTANT, 0.9 PER CENT

| Per Cent<br>of<br>Lecithin | Per Cent<br>of<br>Span 60 | Activity |     |     |     |      |      |      | Neg.<br>Ser. | NaCl |
|----------------------------|---------------------------|----------|-----|-----|-----|------|------|------|--------------|------|
|                            |                           | 1/1      | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 |              |      |
| 0.15                       | 0.1                       | —        | —   | —   | —   | —    | —    | —    | —            | —    |
| 0.15                       | 0.3                       | —?       | ±   | +   | +   | ±    | —?   | —    | —            | —    |
| 0.15                       | 0.6                       | +        | ++  | +++ | +++ | ++   | ±    | —    | —            | —    |
| 0.30                       | 0.1                       | —        | —   | —   | —   | —    | —    | —    | —            | —    |
| 0.30                       | 0.3                       | +++      | +++ | +++ | +++ | ++   | —    | —    | —            | —    |
| 0.30                       | 0.6                       | +        | +++ | +++ | +++ | ++   | —    | —    | —            | —    |
| 0.60                       | 0.1                       | —        | —   | —?  | —?  | —    | —    | —    | —            | —    |
| 0.60                       | 0.3                       | —        | +   | ++  | +   | +    | —    | —    | —            | —    |
| 0.60                       | 0.6                       | —?       | ++  | ++  | ++  | ++   | ±    | —    | —            | —    |

serum. This occurs especially when the amount of the reacting component in the examined substance is small. In this case it probably is a matter of the ratio between the reacting component of the serum and the reacting component in the antigen crystals. This inhibition, however, must not be confused with the characteristic of some sera to inhibit reactions of this type in general, which is independent of, for example, the amount of C-reactive protein in the serum. In the following table (Table 3) an example of inhibition of this type is given: Serum A, which reacts with sitolipin antigen but in which there is definite inhibition in the first dilutions, does not react in the APC test. Serum B is negative in the sitolipin test but positive in the APC test, in which there is no inhibition. By carrying out both tests with an 1 + 1 serum mixture a result is obtained in which inhibition occurs in both tests. This inhibition is transferred to the APC test only in the case that the added sitolipin positive serum originally is inhibitive.

TABLE 3

|             |           | 1/1 | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 |
|-------------|-----------|-----|-----|-----|-----|------|------|------|
| Serum A     | Sitolipin | ±   | ±   | +   | ++  | +++  | +    | —    |
|             | APC       | —   | —   | —   | —   | —    | —    | —    |
| Serum B     | Sitolipin | —   | —   | —   | —   | —    | —    | —    |
|             | APC       | +++ | +++ | ++  | ++  | —    | —    | —    |
| Serum A + B | Sitolipin | ±   | +   | ++  | ++  | +    | —    | —    |
|             | APC       | ±   | +   | ++  | —?  | —    | —    | —    |

Very interesting inhibition phenomena were revealed when we studied fractions and the original substance in isosorbide stearate synthesis. We repeat below the results obtained with different fractions from this synthesis:

TABLE 4  
ACTIVITIES OF DIFFERENT FRACTIONS OF ISOSORBIDE STEARATE SYNTHESIS

| Fraction No.     | Activity |     |     |     |      |      |      | Neg. Ser. | NaCl |
|------------------|----------|-----|-----|-----|------|------|------|-----------|------|
|                  | 1/1      | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 |           |      |
| 50               | —        | —   | —   | —   | —    | —    | —    | —         | +    |
| 51               | —        | —   | —   | —   | —    | —    | —    | —         | +    |
| 52               | ++       | ++  | +++ | +++ | ++   | +    | —?   | —         | —    |
| 53               | ++       | +++ | +++ | +++ | ++   | ±    | —    | —         | —    |
| 50—53            | —        | —   | —   | —   | —    | —    | —    | —         | +    |
| APC              | +++      | +++ | +++ | +++ | ±    | —    | —    | —         | —    |
| 50+52            | —?       | —?  | —?  | —?  | ±    | ±    | ±    | ±         | +    |
| 52 + stear.acid. | ++       | ++  | ++  | ++  | ±    | —    | —    | —         | —    |
| 52 + isosorbide  | +        | +   | ±   | —?  | —?   | —?   | —?   | —?        | —?   |
| Stearic. acid    | —        | —   | —   | —   | —    | —    | —    | —         | —    |

Fraction No. 50, which is closest to distearate, thus inhibits the activity of fraction No. 52 (closest to monostearate). Stearic acid is of no great significance, but isosorbide, if it is contained in the alcoholic antigen, greatly weakens the reaction. The effect of isosorbide on APC antigen was also studied by adding it both to the alcohol solution and to the ready saline antigen. The results were:

|   | 1/1 | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 |
|---|-----|-----|-----|-----|------|------|
| APC .....                                     | +++ | +++ | ++  | —?  | —    | —    |
| APC + isosorbide added to alcohol .....       | —   | —?  | —?  | ±   | —?   | —    |
| APC + isosorbide added to ready antigen ..... | ++  | ++  | ++  | ±   | —    | —    |

Although isosorbide when added to ready antigen has some slightly weakening effect on the reaction, it is of an entirely different magnitude when added to the alcohol solution, as it almost completely cancels the activity of APC-antigen. It is probable that in this case isosorbide disturbs the formation of antigen crystals.



The results obtained in the synthesis of arlitan stearate have already been presented but are repeated below:

| Fraction No. | Activity |     |     |     |      |      |      | Neg.Ser. | NaCl |
|--------------|----------|-----|-----|-----|------|------|------|----------|------|
|              | 1/1      | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 |          |      |
| 70           | —        | —   | —   | —   | —    | —    | —    | —        | ++   |
| 71           | +++      | +++ | +++ | ++  | ±    | —?   | —?   | —        | —?   |

Fraction No. 70 corresponded chiefly to distearate and fraction No. 71 to monostearate, the former being inactive and the latter active. When increasing the concentration of lecithin in the antigen, the arlitan distearate acquired reactivity to C-reactive protein. The same occurred in the sorbitol dipalmitate fraction, which in itself is almost inactive, but not in the fraction corresponding to tripalmitate.

On reviewing the positive reactions given by the various substances in the sera of patients with various diseases hardly any differences were seen in the various substances. The only exceptions were the fractions of trehalose stearate synthesis, which in sera from patients with pulmonary tuberculosis gave results slightly different from those obtained in the APC test.

After Noll (10) had published his studies of the so-called cord factor of the tb bacillus, which he demonstrated to be trehalose-6, -6'-dimycolate, and taking also into consideration the serological results obtained by Tommila (3) with the cord factor, we prepared a substance of this type. Mycolic acid was substituted by a long chain fatty acid of nearly corresponding solubility, which was isolated from beeswax according to Nafzger (11). The «kerotic acid» obtained is, according to Nafzger, a mixture of  $C_{26}$ — $C_{30}$  fatty acids. The melting point of our product was  $+70^{\circ}\text{C}$  and the acid value 140. The acid mixture was esterified with methanol, yielding an ester with an acid value of 7, saponification value of 132 and melting point of  $+55^{\circ}\text{C}$ . Because of the small amount obtained we could not carry the purification further. The methyl «kerotinate» and the trehalose dihydrate were esterified in a ratio corresponding to a diester and the synthesized product was fractionated from ethanol. The synthesis mixture and the fraction precipitating at below  $-10^{\circ}\text{C}$  showed slight affinity to C-reactive protein. Fraction  $\pm 20^{\circ}$ — $\pm 0^{\circ}\text{C}$  and especially fraction  $\pm 0^{\circ}$ — $-10^{\circ}\text{C}$ , which had a saponification value of 77 and cor-

responded closest to trehalose monokerotinate, was completely negative when examined with a serum containing C-reactive protein but nevertheless reacted with some pulmonary tb patients' sera that were negative in the APC test, up to a very high titer, 1/128. We tested with this fraction about 100 sera from pulmonary tb patients, over 10 per cent of which were reactive, many of them up to a dilution 1/16 and more. As controls were tested the same number of sera from patients in the hospital's medical wards, in which positive reactions were obtained in about 4 per cent, the highest titer being 1/16. Since we did not have cord factor available we could not compare these results with the reactions obtainable with cord factor. However, our results point to some similarity to the manner in which the cord factor has been found to behave.

To conclude, we shall compare the results obtained in the present studies with the cord factor features considered by Noll to be prerequisites for its biological activity. These are:

- a) The lipid must be chemically bound to carbohydrate, since neither component alone is not active.
- b) The trehalose component must contain free hydroxyl groups, since methylation or acetylation destroys the activity.
- c) The position of the ester group in the sugar component is important, only carbon 6 esters being active.
- d) Substitution of trehalose by glucose have no effect on the activity, whereas a change in the configuration destroyed the activity.
- e) The structure of the lipid component was not sharply specific.

The activity of the examined substances towards C-reactive protein complied to a very great degree with the requirements stated above; points c and part of d only did not need to be complied with. The conditions for activity presented in the same manner as above would be as follows:

- a) Esterification is necessary, the components in themselves are not active.
- b) Methylation of the hydroxyl groups destroys the activity.
- c) Location of the ester group at carbon 6 is not necessary.

- d) Structure of the hydrophilic component is not specific, but results with both trehalose stearate and trehalose kerotinate point to some possible significance.
- e) Fatty acid chain is not greatly specific, for positive reactions were obtained with both lauric acid and «kerotic» acid. Additionally it was observed that it was not absolutely indispensable for the esters to be monoesters.

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## ACTIVITY OF THE HYPOTHALAMO-NEUROHYPOPHYSIAL NEUROSECRETORY SYSTEM AFTER IPRONIAZID TREATMENT

by

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Earlier investigations have revealed that antidiuresis is obtained when experimental animals are injected exogenically with such biologically active amines as serotonin (11, 1, 26) and catecholamines (12, 8, 13). It seems that, in the case of catecholamines (12, 8, 13) and of serotonin at least (17, 20, 21, 22), the resultant antidiuresis is partially accountable to the stimulating effect of the drug on the hypothalamo-neurohypophysial system, which is the place where, according to the now accepted opinion (for ref. see 29, 3), the posterior pituitary hormones are formed and bound with the neurosecretory material.

The said monoamines have been found to occur in the brain in comparatively great quantity particularly in the hypothalamus (34, 6, 4) which also contains remarkable quantities of monoamino oxidase (4) the enzyme which decomposes them (30, 7). It would therefore appear interesting to study what effects upon the activity of the hypothalamo-neurohypophysial system might result from inhibition of the monoamino oxidase (MAO) by iproniazid, since the quantity of these amines would then be endogenically raised (27, 33, 32, 31, 5).

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## MATERIAL AND METHODS

In the present investigation 50 white male rats weighing 180 g on the average were employed as experimental animals. Twentyfive animals were subcutaneously injected with iproniazid<sup>1</sup> (N<sup>2</sup>-isopropyl-isonicotinic acid hydrazide «Marsilid», Hoffmann-LaRoche) using a dosage of 100 mg/kg (0.1 ml). After 2, 4, 8, 16 and 24 hours had elapsed from the injection the animals were killed five at a time. All animals and five controls were killed during one afternoon. Ten animals were subcutaneously injected with iproniazid in a daily dosage of 100 mg/kg (0.1 ml) during seven days. Ten control animals were given similar subcutaneous injections of 0.1 ml physiological saline daily. The rats could take food and water *ad libitum* during the test; even otherwise they received the same treatment as the other animals in the laboratory. Seven hours after the last injection the animals were killed.

All the animals were killed by rapid decapitation with a pair of scissors. The hypothalamus and hypophysis were immediately excised taking care to maintain the connecting hypophyseal stalk intact. They were fixed in Bouin's fluid during seven days, treated in the usual manner and embedded in paraffin. The hypothalamus-hypophysis blocks were sectioned in the sagittal plane serially into cuts of 7 micra, two parallel series being taken at a mutual spacing of 50 micra from the lateral part where the supraoptic nucleus is located, while all cuts from the central part of the hypothalamus, comprising the median eminence and its surrounding region, were taken upon a slide as one single series. For the demonstration of the neurosecretory material (N.S.M.), all cuts from the central part of the hypothalamus and those of the first series from the lateral part were stained with Gomori's (15) aldehyde-fuchsin (A—F) in the modification presented by Landing *et al.* (23). The cuts of the second series from the lateral part were stained with methyl green-pyronin for measurement of the nucleus and nucleolus.

The relative quantity of N.S.M. in the perikaryon of the neuron of the supraoptic nucleus (S.O.N.) and the paraventricular nucleus (P.V.N.), in the region of the supraoptico-hypophyseal tract and

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<sup>1</sup> Kindly supplied by Hoffman-La Roche, Basel.

in the infundibular process was assessed visually by a scale from 0 to 5. The same procedure was also applied in estimating the number of the A—F stained nerve fibers in the ventral wall of the median eminence, running toward the capillaries of the hypophyseal portal vessels, as well as the quantity of material stainable with A—F in their neighbourhood. This material has been found to belong to the hypothalamo-neurohypophyseal system both with respect to its histochemical nature and to its functional significance (28). All assessments were made fully at random, the examiner being unaware of the group to which the cuts from each animal belonged.

Measurements of the nucleus and nucleolus were carried out with the ocular micrometer under magnification  $1600 \times$ . 100 cells each were measured from the S.O.N. of the first five control animals and of the first five animals in the chronic test group. Only cells of the S.O.N. were subjected to such measurements because it has been shown that the changes in the nucleus and nucleolus of the neurons both in the S.O.N. and in the P.V.N. follow a linear relation when the hypothalamo-neurohypophyseal system is stimulated (25, 10, 24, 2). The largest and smallest diameter of the nucleus and the diameter of the nucleolus were measured and the volumes were calculated from these values in the usual manner (14).

#### RESULTS AND DISCUSSION

*Acute Experiments.* — In these acute experiments no difference could be observed in the relative amount of the NSM in any part of the hypothalamo-neurohypophyseal system. This observation is in agreement with the earlier investigations that acute stress is not sufficient to cause potential neurosecretory response (18).

*Chronic Experiments.* — Table I shows the relative quantity of N.S.M. in the various parts of the hypothalamo-neurohypophyseal system. Although it can be seen that the animals treated with iproniazid had somewhat less N.S.M. than the controls, particularly in the infundibular process, the differences were not of an order of magnitude which would justify the claim that they are statistically significant. With respect to the A—F material occurring around the capillaries of the hypophyseal portal vessels in the ventral wall of the median eminence and to the A—F stained

TABLE 1

THE RELATIVE AMOUNT OF NEUROSECRETORY MATERIAL IN THE SUPRAOPTIC (S.O.N.) AND PARAVENTRICULAR (P.V.N.) NUCLEUS, AROUND THE HYPOPHYSIAL PORTAL VESSELS (H.P.V.) AND IN THE TRANSVERSE NERVE FIBRES (T.N.F.) IN THE VENTRAL WALL OF THE MEDIAN EMINENCE (M.E.), AND IN THE INFUNDIBULAR PROCESS (I.P.) IN THE CHRONIC TEST SERIES

|                 | Number of Animals | S.O.N.                 |                | P.V.N.     |                | M.E.              |                |            |                | I.P.       |                |
|-----------------|-------------------|------------------------|----------------|------------|----------------|-------------------|----------------|------------|----------------|------------|----------------|
|                 |                   | Arb. Units             | p <sup>2</sup> | Arb. Units | p <sup>2</sup> | Around the H.P.V. |                | T.N.F.     |                | Arb. Units | p <sup>1</sup> |
|                 |                   |                        |                |            |                | Arb. Units        | p <sup>2</sup> | Arb. Units | p <sup>2</sup> |            |                |
| Control . . . . | 10                | 3.8 ± 0.1 <sup>1</sup> |                | 3.7 ± 0.2  |                | 3.6 ± 0.2         |                | 3.8 ± 0.3  |                | 4.0 ± 0.3  |                |
| Iproniazid . .  | 10                | 3.8 ± 0.2              | >0.05          | 3.6 ± 0.3  | >0.05          | 3.6 ± 0.3         | >0.05          | 3.7 ± 0.3  | >0.05          | 3.7 ± 0.2  | >0.05          |

TABLE 2

THE NUCLEUS AND NUCLEOLUS VOLUMES IN THE SUPRAOPTIC NUCLEUS OF CONTROL AND TEST ANIMALS

|                      | Number of Cells | Nucleus                  |                | Nucleolus      |                |
|----------------------|-----------------|--------------------------|----------------|----------------|----------------|
|                      |                 | μ <sup>3</sup>           | p <sup>2</sup> | μ <sup>3</sup> | p <sup>2</sup> |
|                      |                 |                          |                |                |                |
| Control . . . . .    | 500             | 386.7 ± 5.9 <sup>1</sup> |                | 7.2 ± 0.2      |                |
| Iproniazid . . . . . | 500             | 415.2 ± 12.0             | <0.05          | 7.4 ± 0.2      | >0.05          |

<sup>1</sup> Standard error.

<sup>2</sup> Compared with controls.

transverse nerve fibres running toward these capillaries, too, no significant difference was established between the test and control groups.

Table 2 contains the nucleus and nucleolus volumes derived from the measurements. The iproniazid-treated animals have greater volume of the nucleus as well as the nucleolus but only for the nucleus volume there is a statistically significant difference between the two groups.

Earlier investigations have revealed that increase in volume of the nucleus and nucleolus of the neurosecretory neurons is a good criterion for increased activity (25, 10, 24, 2). The results obtained in the present work with respect to the nucleus and nucleolus volume are thus in support of increased activity of the hypothalamo-neurohypophyseal system in the rats which were given iproniazid in the chronic experiments. Although there were no distinct differences in the quantity of N.S.M., this does not actually speak against the idea of increased activity; it has been observed in numerous previous connections that the quantity of N.S.M. alone does not always provide a positive basis for inferences concerning the activity of the hypothalamo-neurohypophyseal system (*e.g.* 19).

Since iproniazid increases the quantity of serotonin and catecholamines in the brain (27, 33, 32, 31, 5) by inhibiting the action of monoamino oxidase (30, 7), its effect on the hypothalamo-neurohypophyseal system might be based on this action. There is reason for such an assumption on the strength of the recent observations, according to which serotonin stimulates the hypothalamo-neurohypophyseal system (17, 20, 21, 22). It is possible that the increased quantity of catecholamines also contributes to the effect, although the results relating to their influence on the activity of the hypothalamo-neurohypophyseal system are controversial (12, 8, 13, 16, 9).

Of course, the results obtained here may be accountable by the stimulating effect of iproniazid on the brain, which must not necessarily be associated with the increased serotonin and catecholamine quantities. Further research is required before the mechanism of the stimulating effect of iproniazid on the hypothalamo-neurohypophyseal system can be positively clarified.



## SUMMARY

Using rats as experimental animals, the effect of acute and chronic iproniazide treatment on the neurosecretory hypothalamo-neurohypophyseal system has been investigated. In the acute experiments no positive effect was obtained with iproniazide 2, 4, 6, 8 and 24 hours after the injection. However, the chronic test of 7 days' duration produced stimulation of the system; at the same time a statistically significant increase in volume of the nucleus in the neurons of the nucleus supraopticus was noted. With respect to the quantity of neurosecretory material in the different parts of the hypothalamo-neurohypophyseal system no significant differences were established between the test and control animals. The possible mechanism of action has been discussed.

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## METABOLISM OF PROGESTERONE BY THE LIVER OF FETAL AND NEW-BORN GUINEA PIG

by

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It has been shown by Forbes (2) that the umbilical arterial blood has greater progesterone activity than venous blood. He claims that the fetus secretes progesterone and that the placenta destroys it. In a recent work by Solomon *et al* (11) it was shown that the fetal adrenals which produce significant amounts of progesterone can metabolize it to inactive metabolites as regards to the protective action of progesterone on the uterine contractivity.

The hormonal theories concerning the onset of labor have been a matter of lively discussion (9). Some authorities think that the role of progesterone does not throw further light on the problem since the excretion of pregnandiol does not change during the time of the onset of parturition according to their observations. The progesterone produced by the placenta is, however, considered to pass first through the fetus (16). According to the present view progesterone is thought to protect the uterus against the contractive effects of the oxytocin substances. For the moment it was considered to be interesting to study the effect of the fetal liver to metabolise progesterone. Similar studies have been performed with adult rabbit and rat liver (13, 14, 15). In the present work fetal guinea pigs were used.

### MATERIAL AND METHODS

The series comprised 6 new-born guinea pigs and 7 fetus. The studies were performed *in vitro* conditions.

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*Tissue Specimens.* — The livers of the fetus and the new born were removed immediately after the animals were killed by a blow on the head. This slices were then taken of the tissue and transferred in the incubation mixture in Warburg flasks.

*Progesterone Substrate.* — 10 mg of crystallized progesterone (Sigma Co) was dissolved in 1 ml propylene glykol according to Sie and Fishman (10) and Lehtinen *et al.* (7).

*Incubation.* — The tissue slices were incubated in the Warburg apparatus at 37°C over a period of 90 minutes (12). The incubation mixture contained the following substances:

1) 0.90% NaCl, 2) 1.15% KCl, 3) 1.22% CaCl<sub>2</sub>, 4) 2.11% KH<sub>2</sub>PO<sub>4</sub>, 5) 3.82 MgSO<sub>4</sub> · 7 H<sub>2</sub>O, 6) 0.1 M phosphate buffer, pH 7.4, (17.8 g Na<sub>2</sub>HPO<sub>4</sub> · 2 H<sub>2</sub>O + 20 ml HCl 1 N, diluted to 1000 ml with H<sub>2</sub>O), in the following ratios 100:4:3:1:1:12. To 40 ml of this mixture was added 20 ml of 0.15 M KCl-solution, 0.2960 g of nicotinamide and 0.0277 g DPN.

3 ml of this final mixture were transferred to the Warburg flasks. 0.050 ml (500 γ) of the progesterone in the propylenglykol was added to it after which the tissue slices (dry weight 5–10 mg) were placed into the incubates. The flasks were gased with 5 per cent CO<sub>2</sub> in O<sub>2</sub>. Control runs were performed in which the incubation mixture without the tissue samples and also vice versa the same with tissue slices but without progesterone.

*Sampling.* — After the incubation the tissue slices were removed and dried at 90° C for later weighing. The contents of two Warburg flasks were combined.

*Deproteinization.* — This was performed by heating the incubation mixtures at 60° C for 3 minutes and the precipitated proteins were removed by centrifugation.

*Extraction of Free Progesterone.* — This was carried out from the deproteinized solution with methylenchloride (3 × 10 ml).

*Extraction of Glucuronides.* — The water phase left after methylenchloride extraction was made 2 N with HCl and the glucuronides were extracted with ethylacetate (3 × 10 ml). The ethylacetate was removed in vacuum.

*Hydrolysis.* — To the ethylacetate residue were added acetate buffer (2 ml, 0.1 M, pH 4.5) and β-glucuronidase entzyme preparation (2 mg Worthington Biochem. Corp.). The hydrolysis was carried out over a period of 24 hours at 37°C.

*Extraction of Progesterone Liberated by Hydrolysis.* — After the hydrolysis with  $\beta$ -glucuronidase the incubation solution was re-extracted with methylenchloride ( $3 \times 7$  ml). Methylenchloride was removed in vacuum.

*Estimation of Progesterone.* — Unchanged progesterone present after the incubation was determined from the evaporate residue of methylenchloride extract with 2, 4-dinitro-phenylhydrazid (3). Also the methylenchloride solution extracts taken after the hydrolysis were similarly analysed for their free progesterone contents.

*Estimation of Pregnandiol.* — In addition to the abovementioned progesterone analyses of the methylenchloride extracts taken after the hydrolysis also the free pregnandiol was determined from these by using the  $\text{KHSO}_3 - \text{H}_2\text{SO}_4$ -reagent (1).

*Paperchromatography.* — Progesterone and pregnandiol were also chromatographically identified from the evaporate residues. Progesterone spots were detected with m-dinitrobenzine (4, 6) and pregnandiol with Fast Black Salt K-solution (5).

*Control Analyses.* — The Warburg-incubations were performed without progesterone which was then added after the incubation. From here on the procedure were as above. Also the same was made without tissue slices.

The recovery values obtained in these control experiments were used as basis for later comparison of the metabolised progesterone.

## RESULTS

Table 1 shows the amounts of unchanged progesterone and the amount of pregnandiol-type metabolites produced by the incubation.

According to the recovery test the real reference value is 555  $\gamma$ . It can be noted that about half of the progesterone is thus metabolized. Of this only about one tenth could be identified as a pregnandiol-type metabolite. This observation has also been verified by the paper chromatography method.

In the table the fetus are listed in their weight order but no significant tendency for a decrease in the unchanged progesterone towards the parturition can be detected. Likewise is the somewhat smaller amount unmetabolized progesterone in the new borns without significance. It should be pointed out, however, that in the

TABLE 1

| Fetus No. | Unchanged<br>Progesterone<br>$\gamma$ | Pregnandiol-type<br>Metabolites $\gamma$ |
|-----------|---------------------------------------|--|
| 1         | 275                                   | 27.8                                     |
| 2         | 289                                   | 36.1                                     |
| 3         | 280                                   | 16.8                                     |
| 4         | 277                                   | 17.2                                     |
| 5         | 271                                   | 24.5                                     |
| 6         | 306                                   | —  |
| 7         | 274                                   | 46.1                                     |
| mean      | 280                                   | 30.3                                     |
| New-born  |                                       |  |
| 1         | 229                                   | 39.1                                     |
| 2         | 218                                   | 33.0                                     |
| 3         | 223                                   | 36.3                                     |
| 4         | 192                                   | 30.4                                     |
| 5         | 281                                   | 32.9                                     |
| 6         | 285                                   | 39.5                                     |
| mean      | 231                                   | 35.3                                     |

newborn group the amount of pregnandiol-type metabolites is also smaller than in the smallest fetus.

#### DISCUSSION

According to the results it is obvious that the fetus liver is capable to metabolize progesterone. Since the final identification of the metabolites is not made it is not possible to claim whether these are without the progestational protective activity against the oxytocine induced uterine contraction. According to the literature only man and rabbit excrete progesterone as a pregnandiolglucuronide (8). Neither have we been able in the present work to find significant amounts of pregnandiolglucuronide, and the specificity of it is also not quite sure in spite of the paperchromatography control. This latter indicate that pregnandiol or a close derivative of it is in question. These observations support the view that the guinea pig belongs to those species which excrete progesterone mainly as another metabolite than pregnandiolglucuronide.

Even though it has been possible to show that the fetal guinea pig liver is able to metabolize progesterone no abrupt increase in this

metabolic gradient is evident at the time of birth. It might be that the adult liver is able to metabolize more rapidly than during fetal life. Quantitatively the fetal liver naturally grows and with this the total metabolic capacity. Another precipitating factor for the disturbance of the equilibrium between progesterone and oxytocin activities is the possible increase in the liberation of oxytocin.

Quantitative determination of these factors is now under progress in this laboratory.

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## AN ATTEMPT TO PROVOKE RHEUMATOID FACTOR-LIKE PHENOMENON IN RABBIT SERUM BY REPEATED INTRAVENOUS INJECTIONS OF PAPAIN

by

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The primary nature of the agglutination activating factor («rheumatoid factor»), present in 60–80% of rheumatoid sera, is still unknown.

It has been previously reported that papain protease, given intravenously, has a digestive proteolytic effect on the cartilage matrix, and on the cardiac and skeletal muscle (4, 10, 6, 7).

Could the substances, liberating *in vivo* by proteolysis, act as autoantigens and could a rheumatoid factor-like protein appear in the serum during this possible autoimmunological phenomenon? This was the idea which inspired us to the experiments reported in this paper.

### MATERIAL AND METHODS

The experiments were performed on five rabbits.

One ml of 1% water solution of crude papain was injected into the lateral ear vein of the rabbits twice a week during six weeks. The papain solution was sterilized by filtration through the Seitz' filter and a fresh solution was prepared weekly.

The collapse of rabbit ears, reported by Thomas (10), was seen in one rabbit only during the sixth week. Since we did not see

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this phenomenon, we tried to produce it by giving 5 ml of 1% papain solution intravenously to one of the animals on the fifth week of the experiment. This rabbit died 2 hours after the injection with symptoms of anaphylactoid shock. All test animals began to look tired and ill during the fifth week and two rabbits died during the sixth week. The organs of three died animals were grayish and the presence of proteolysis was verified by microscopic examination. The two rabbits which were alive after the whole experiment, looked later to be completely well without any joint symptoms.

The blood samples were taken weekly during the whole experiment. The two rabbits still alive after the last papain injection were bled one week after the last injection, too.

The blood samples were subjected to the following tests:

*The precipitation test* for the detection of the antipapain antibodies, possibly developing during these repeated papain injections. The results were read after one half hour's incubation in 37°C.

*The modified latex fixation test* was performed using acrylplast suspension (AB Bofors Nobelkrut Akrylplastpulver). The size of these acrylplast particles is 0.5 microns; they were suspended into physiological saline so that the final content of the suspended particles was 11%. (The reliability of these acrylplast particles in the detection of rheumatoid factor in human serum has been confirmed by Carlson (1).) The particles were sensitized with rabbit's gammaglobulin, prepared by us using the ammoniumsulphate technic. The gammaglobulin concentration of the solution used was 6.5%. The final acrylplastgammaglobulin reagent was prepared as follows:

0.4 ml of rabbit's gammaglobulin solution (25 mg of gammaglobulin),

0.5 ml of acrylplast suspension,

4.1 ml of glycine buffer, pH 8.2.

One drop of rabbit serum was added into 1 ml of glycine buffer. One drop of this serum dilution was mixed with one drop of acrylplast-gammaglobulin reagent on an object slide and the results were read as in the usual latex fixation test.

*The sensitized sheep red cell agglutination test* was performed according to the modification which has been introduced by Svartz and Schlossmann (8).

## RESULTS

During the 5th—6th week of the experiment it was possible to detect precipitins against 1% crude papain solution in the sera of all five rabbits. During the first four weeks of experiment the precipitation tests were negative.

All tests performed for the detection of rheumatoid factor-like phenomenon (an agglutination test using acrylplast suspension sensitized with rabbit's gammaglobulin as reagent, and the sensitized sheep red cell agglutination test) were negative during the whole experiment and also after it.

## DISCUSSION

Svartz and Schlossman (9) wrote: »— it is probable that the specific haemagglutinating substance in serum taken from patients with rheumatoid arthritis is formed as a result of enzymatic influence upon the collagen tissue in the organism». This hypothesis is very logical since we know, for example, that many microorganisms, especially streptococci, may produce *in vivo* tissue digesting proteolytic enzymes (2, 3). On the other hand, it has been presented (5) that the rats with experimental infectious arthritis, produced by a strain of *Streptobacillus moniliformis*, developed elevated titers in the bentonite flocculation and sheep cell agglutination tests.

In our experiments using repeated papain injections as proteolytic agent it was not possible to provoke rheumatoid factor-like phenomenon in rabbit serum. This, in our opinion, does not exclude the abovementioned hypothesis on the developing mechanism of the rheumatoid factor. It may be that the rabbit is not the right object for experiments of this kind and papain not the right enzyme.

## SUMMARY

1) Five rabbits were subjected to repeated intravenous injection of 1 ml of 1% crude papain solution twice a week during six weeks.

2) An agglutination test using cerylplast suspension sensitized with rabbit's gammaglobulin as reagent and the sensitized sheep red cell agglutination test performed according to the method of Svartz and Schlossmann did not become positive during the experiment or after it.

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## REACTION OF THE RHEUMATOID FACTOR WITH HUMAN SPECIFIC PRECIPITATES

### I

ADSORPTION OF THE RHEUMATOID FACTOR TO AND ELUTION FROM  
AUTOGENOUS AND ISOGENOUS RHEUMATOID DIPHThERIA TOXOID—  
ANTITOXIN PRECIPITATES

by

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The reactivity of the Rheumatoid Factor (RF) with a variety of agglutinating antigen-antibody systems is well established. Of the precipitating systems hitherto studied, all specific precipitates containing rabbit antibody have been found to absorb nitrogen and RF activity from rheumatoid sera (2, 3, 10). A number of other specific precipitates, among them diphtheria toxoid-human antitoxin precipitates did not seem to possess such reactivity (10).

Strangely enough, the report published by Vaughan in 1956 (10) is still the only one dealing with the reactivity of the RF with specific precipitates of human origin. For various reasons, such as the possibility of the RF being an auto-antibody directed against the patient's own gamma globulin, the availability for immunochemical research of a precipitating system of human origin would be most desirable.

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In the present work, such precipitates composed of diphtheria toxoid and rheumatoid antitoxin were found to adsorb even the patient's own RF, which subsequently could be recovered by elution of the washed precipitates at pH 5.

#### TECHNIQUE

*Agglutination of Sensitized Sheep Cells.* — The method described elsewhere (1) for the Waaler-Rose test was followed. All rheumatoid sera were first absorbed with normal sheep cells. For sensitization of the cells,  $\frac{1}{3} \times$  MAD of a rabbit anti-sheep cell amboceptor was used.

*Agglutination of Sensitized O Rh Positive Cells.* — The method of Harboe and Lundewall (5) was followed. Differing from it, the reagents were first measured into 100 mm  $\times$  12 mm test tubes. Following incubation for one hour at room temperature, one drop of each mixture was placed within the rings of a glass slide. The readings were made immediately thereafter microscopically under low magnification.

The anti-D serum «Ripley» was obtained through the courtesy of Drs. Marion Waller and J. Vaughan. The anti-D serum «201/60» was found by us among some two hundred sera containing incomplete antibodies and obtained through the courtesy of Dr. H. R. Nevanlinna, chief of the Central Blood Bank of the Finnish Red Cross.

*Latex Agglutination.* — The method of Singer and Plotz (9) was followed: «1.64 M ammonium sulphate fractionation of rheumatoid arthritis serum using latex-commercial gamma globulin». In testing eluates the ammonium sulphate fractionation was omitted. The polystyrene latex particles were obtained commercially from Difco Laboratories («Bacto-Latex 0.81»), and the human gamma globulin from AB. Kabi, Stockholm.

*Preparation of Specific Precipitates.* — For each rheumatoid antitoxin, the approximate equivalence point was determined by preliminary precipitation tests as described by Kabat and Mayer (6) and by antitoxin assay in the guinea pig skin. Diphtheria toxoid was added to undiluted rheumatoid antitoxin in the region of slight antigen excess. The mixture was incubated in the water-bath at  $+37^{\circ}\text{C}$ . for one hour and kept overnight at  $+4^{\circ}\text{C}$ . in the refrigerator. The precipitate was spun down, washed three times with a large volume of chilled saline, and suspended in saline to make the volume of the original antitoxic serum. As preservative, 'Merthiolate' (trade-mark of Eli Lilly and Co. for Thimerosal) 1:5000 was added to the precipitate suspension. Highly purified fluid diphtheria toxoid, containing 1800 Lf/ml, was obtained through the courtesy of Dr. P. Halonen, chief of the microbiological department of Lääketehdas Orion, Helsinki.

*Absorption with Specific Precipitates.* — Equal volumes of precipitate suspension and undiluted rheumatoid serum were mixed. Following incubation overnight in the refrigerator, the precipitate was separated by centrifugation. From the second absorption onward, packed precipitate

instead of precipitate suspension was used. Before performance of the Waaler-Rose and latex tests with absorbed sera, portions of the sera were inactivated by keeping them in a water-bath at  $+56^{\circ}\text{C}$ . for 30 minutes. Non-inactivated portions were employed when testing for agglutination of sensitized O Rh positive cells.

*Elution of Specific Precipitates.* — The antitoxic precipitate obtained from one milliliter of rheumatoid serum was washed three times with a large volume of chilled saline. The saline from the third washing showed in some instances a slight latex agglutinating activity (titers 40–80), whereas agglutination of sensitized sheep and O Rh positive cells gave consistently negative results. Some spontaneous precipitate formation was seen in the absorption control tubes of the sera K.O. and H.K. containing rheumatoid serum alone diluted 1:2 with saline. Eluate made of the spontaneous precipitate K.O. gave a Waaler-Rose titer of 16 and a latex titer of 160, but no measurable agglutinating activity against sensitized O Rh positive cells. The eluate made of the minute spontaneous precipitate H.K. yielded no measurable RF activity whatsoever.

One and a half milliliter of an acetate buffer pH 5.0 was added to the washed antitoxic precipitate. The suspension was kept 1–1.5 hours at room temperature and occasionally agitated gently by hand. The precipitate was spun down and the supernatant separated. The whole procedure was repeated. The two supernatants were combined, neutralized with a slight excess of tris(hydroxymethyl)aminomethane (Sigma Chemical Co.), and adjusted to ionic strength 0.15 with distilled water. This resulted in a final volume four times that of the original rheumatoid antitoxic serum. Eluates were not inactivated before performance of the Waaler-Rose and latex tests.

*Gm Grouping.* — The technique described by Harboe and Lundewall (5) was followed. As the rheumatoid antitoxic sera did not agglutinate O Rh positive cells sensitized with the particular anti-D serum (201/60) employed for Gm grouping, heating of the rheumatoid sera at  $+63^{\circ}\text{C}$ . could be omitted.

*Immunization with Diphtheria Toxoid.* — Rheumatoid subjects showing no immediate type wheal and erythema reaction to the Schick control solution were selected for immunization. Schick tests were not performed. The patients received in all six injections, 0.25 — 0.5—0.5—0.5—0.5—0.5 ml, of a commercial alum-precipitated toxoid, stated by the manufacturer (Lääketehtas Orion) to contain at least 50 Lf/ml. The injections were given by the intramuscular route once a week. The blood specimens were taken one week following the sixth injection.

*Rheumatoid Sera.* — Sera of four different subjects were employed in this work. All of the subjects fulfilled the American Rheumatism Association criteria (8) for «definite rheumatoid arthritis».

Two of the sera, designed «rheumatoid antitoxins», originated from subjects A.S. (No. R-2786/60) and H.K. (No. R-2850/60) who were immunized with diphtheria toxoid. The third serum, T.A. (No. R-2045/60), was selected because of its capacity, not present in the other three sera, to

agglutinate O Rh positive cells sensitized with a certain anti-D serum 201/60. The fourth serum, K.O. (No. R-21/60), had an exceptionally high RF titer, measurable by three different techniques.

All the titers given in this study are expressed as reciprocals of the dilutions, calculated from the total volume of the reagents in the tube.

*Assay of Diphtheria Antitoxin.* — The technique of the intracutaneous rabbit test (4) was followed with the exception that, for reasons of economy, guinea pigs were used instead of rabbits. The standard antitoxin was obtained from the Danish State Institute, Copenhagen. The toxin was obtained through the courtesy of Dr. P. Halonen of Lääketehdas Orion.

*Nitrogen Analyses.* — The Markham modification (7) of the micro-Kjeldahl method was followed, using a Scholander burette.

#### RESULTS AND DISCUSSION

Two rheumatoid subjects containing RF in their circulation were immunized with diphtheria toxoid. Both belonged to the serum group Gm (a+). Specific toxoid-rheumatoid antitoxin precipitates (T-RATP) were then prepared in the region of slight antigen excess by adding 0.3 ml (67 Lf) of toxoid per milliliter of rheumatoid antitoxin. Thus, specific precipitates containing 207 and 210  $\mu$ g of nitrogen per milliliter of serum were obtained from the two sera H.K. and A.S., respectively. The washed precipitates were subjected to elution procedures at pH 5. As seen from table 1,

TABLE 1  
ELUTION OF THE RHEUMATOID FACTOR FROM PATIENT'S OWN SPECIFIC  
PRECIPITATE

|   | Rheumatoid Serum | Titer of       |                                    |                        |
|---|------------------|----------------|------------------------------------|------------------------|
|   |                  | Original Serum | Serum after Removal of Precipitate | Elate from Precipitate |
| Agglutination of sensitized sheep cells (Waler-Rose)              | A.S.             | 512            | 256                                | 128                    |
|   | H.K.             | 256            | 128                                | 64                     |
| Agglutination of latex particles coated with human gamma globulin | A.S.             | 40960          | 10240                              | 2560                   |
|   | H.K.             | 10240          | 1280                               | 640                    |
| Agglutination of O Rh + cells sensitized with anti-D Ripley       | A.S.             | 10240          | 2560                               | 640                    |
|   | H.K.             | 320            | 40                                 | 320                    |

RF activity, measurable by three different techniques, could be eluted from both rheumatoid antitoxic precipitates. Attachment of the RF to the T-RATP also was suggested by the decreased RF activity of the rheumatoid sera after removal of the specific precipitates.

The capacity of the rheumatoid precipitates to adsorb RF, in spite of attachment of the patient's own RF to the T-RATP being formed, was not completely lost, as shown by first treating the rheumatoid antitoxic precipitate with a high titer rheumatoid serum of a non-immunized subject, and then eluting the separated washed precipitate. More RF activity could be eluted from such precipitates than from untreated ones, as seen in table 2. This

TABLE 2  
REACTION OF A HIGH TITER RHEUMATOID SERUM K.O. WITH RHEUMATOID SPECIFIC PRECIPITATE H.K.

|   | Titer of            |   |  |  |
|---|---------------------|---|--|--|
|   | Original Serum K.O. | Serum K.O. Absorbed with Precipitate H.K. | Eluate from Precipitate H.K. Treated with Serum K.O. | Eluate from Untreated Precipitate H.K. |
| Agglutination of sensitized sheep cells (Waler-Rose)              | 8192                | 4096                                      | 2048   | 64                                     |
| Agglutination of latex particles coated with human gamma globulin | $\geq 327680$       | $\geq 327680$                             | 10240  | 640                                    |
| Agglutination of O Rh + cells sensitized with anti-D Ripley       | $> 10240$           | $\geq 10240$                              | 2560   | 320                                    |

could also be shown by taking advantage of the observed circumstance that only few rheumatoid sera are able to agglutinate O Rh positive cells sensitized by a certain anti-D serum 201/60. As neither of the sera of the immunized rheumatoid subjects did so, this agglutinating system could be utilized as a marker of the RF of a third rheumatoid serum possessing the agglutinating capacity in question. As shown in table 3, the RF of this particular serum



TABLE 3

AGGLUTINATION OF O Rh POSITIVE CELLS SENSITIZED WITH ANTI-D 201/60 BY THE RF OF A SELECTED RHEUMATOID SERUM T.A.

|  | Dilution of Rheumatoid Serum T.A. |     |     |     |     |     |      |      |      |                |
|--|-----------------------------------|-----|-----|-----|-----|-----|------|------|------|----------------|
|  | 20                                | 40  | 80  | 160 | 320 | 640 | 1280 | 2560 | 5120 | Saline Control |
| Original serum T.A.                                      | +++                               | +++ | +++ | +++ | +++ | ++  | ++   | +    | (+)  | —              |
| Serum T.A. absorbed with rheumatoid precipitate H.K. . . | —                                 | +   | +   | +   | +   | (+) | —    | —    | —    | —              |
| Eluate from precipitate H.K. treated with serum T.A. . . | +++                               | +++ | +++ | +++ | +++ | +   | —    | —    | —    | —              |
| Eluate from untreated precipitate H.K. ....              | —                                 | —   | —   | —   | —   | —   | —    | —    | —    | —              |

T.A. was adsorbable to and elutable from the rheumatoid antitoxic precipitate H.K. containing autogenous RF.

From tables 4 and 5 it can be seen that not even four consecutive absorptions with rheumatoid antitoxic precipitates removed all of the RF activity from serum. As shown in table 5, a definite prozone effect was discernible in the agglutination of sensitized O Rh positive cells by absorbed sera. No such effect was seen in the agglutination of sensitized sheep cells and latex particles.

TABLE 4

EFFECT OF FOUR ABSORPTIONS WITH RHEUMATOID SPECIFIC PRECIPITATES ON THE RF ACTIVITY OF THREE RHEUMATOID SERA

| Rheumatoid Serum | Rheumatoid Precipitate Used for Absorption | Agglutination of Sensitized Sheep Cells |                  | Agglutination of Coated Latex Particles |                  | Agglutination of Sensitized O Rh + Cells |                  |
|------------------|--|---|------------------|---|------------------|--|------------------|
|                  |  | Before Absorption                       | After Absorption | Before Absorption                       | After Absorption | Before Absorption                        | After Absorption |
| A.S.             | A.S.<br>H.K.                               | 512                                     | 128<br>32        | 40960                                   | 320<br>320       | 10240                                    | 2560<br><40      |
| H.K.             | A.S.<br>H.K.                               | 256                                     | 64<br>32         | 10240                                   | 320<br>320       | 320                                      | <40<br><40       |
| T.A.             | A.S.<br>H.K.                               | 1024                                    | 128<br>64        | 20480                                   | 5120<br>1280     | 10240                                    | 2560<br>320      |

TABLE 5  
EFFECT OF FOUR ABSORPTIONS WITH RHEUMATOID SPECIFIC PRECIPITATES ON THE RF ACTIVITY OF THREE RHEUMATOID SERA

| Rheuma-<br>toid<br>Serum | Rheuma-<br>toid<br>Precipitate<br>Used for<br>Absorption | Agglutination of O Rh + Cells<br>Sensitized with anti-D Ripley |    |     |     |     |      |      |      |       |                   |
|--------------------------|--|--|----|-----|-----|-----|------|------|------|-------|-------------------|
|                          |  | 40   | 80 | 160 | 320 | 640 | 1280 | 2560 | 5120 | 10240 | Saline<br>Control |
| A.S.                     | None   | ++   | ++ | ++  | ++  | ++  | ++   | ++   | ++   | +     | —                 |
|                          | A.S.   | —  | +  | ++  | ++  | ++  | ++   | ++   | —    | —     | —                 |
|                          | H.K.   | —  | —  | (+) | —   | —   | —    | —    | —    | —     | —                 |
| H.K.                     | None   | +  | +  | +   | +   | —   | —    | —    | —    | —     | —                 |
|                          | A.S.   | —  | +  | +   | —   | —   | —    | —    | —    | —     | —                 |
|                          | H.K.   | —  | —  | —   | —   | —   | —    | —    | —    | —     | —                 |
| T.A.                     | None   | ++   | ++ | ++  | ++  | ++  | ++   | ++   | ++   | ++    | —                 |
|                          | A.S.   | +  | +  | +   | +   | +   | +    | +    | +    | +     | —                 |
|                          | H.K.   | —  | +  | +   | (+) | (+) | —    | —    | —    | —     | —                 |

Thus, the capacity of rheumatoid antitoxic precipitates to absorb RF activity from serum was inferior to that of a rabbit antitoxic precipitates and of Ea—rabbit anti-Ea precipitates, when measured by the Waaler-Rose technique using sensitized sheep cells. In unpublished experiments we found that absorption with 115  $\mu$ g N of Ea—rabbit anti-Ea precipitate removed all of the Waaler-Rose activity of serum T.A., whereas four absorptions with a total of 828  $\mu$ g N of rheumatoid antitoxic precipitate did not accomplish this, as shown in the present work.

The behavior of the RF in the experiments described above suggests an equilibrium reaction between the RF and the reactant existing bound in the antibody portion of the precipitate and free in the surrounding human serum milieu. This view is in accordance with the opinion held by Vaughan (10).

#### SUMMARY

It has been shown that the Rheumatoid Factor (RF) can be adsorbed to and eluted at pH 5 from the patient's own specific precipitates, consisting of diphtheria toxoid and autogenous antitoxin. Rheumatoid antitoxic precipitates also reacted with the RF of other, non-immunized patients. This could be demonstrated by three different techniques, *i.e.*, agglutination of sensitized sheep cells, of sensitized O Rh positive cells, and of latex particles coated with human gamma globulin.

The behavior of the RF suggested an equilibrium reaction between the RF and its reactant existing bound in the antibody portion of the specific precipitate and free in the surrounding human serum milieu.

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## THE USE OF HEPARINOID IN SCLEROTIC DEGENERATION OF THE FUNDUS OCULI

### EXPERIMENTAL STUDIES

by

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(Received for publication November 30, 1960)

In 1953 Amsler reported a case in which incipient changes disappeared in 6 weeks when treated with vitamin, placenta and vasodilator (1). Several reports have been published on the effects of vasodilators in cases of fundal degeneration (3, 8, 9). The drug chiefly used in recent times in the treatment of macular degenerations is heparin (4, 5, 6, 7, 9). The patients are given vitamins A and E, hydergin and PH 203 in combination with heparin (9).

The method of dosage is perhaps the most serious drawback of heparin therapy. Patients find it a nuisance to submit to intravenous injections for months or even years. This has led to attempts to develop drugs which can be administered orally. The advantage of such agents, *e.g.* heparinoids, is the greater convenience of dosing. Heparin is used for its ability to normalise blood lipoids. Even relatively small doses which have no notable anticoagulative effect have a considerable although transient hypolipemic influence. The «clearing» effect of heparin is more distinct in normal patients than in arteriosclerotic cases (2).

Heparinoids are agents which exert a heparin-like plasma-clearing and antilipemic affect either together with or without an anticoagulant effect. Some heparinoids have a relatively good hypolipemic effect but produce no notable effect on the coagulation mechanism of the blood.

Ateroid is a heparinoid made from the duodenal mucosa. It is by nature a heparin-like polysaccharide which contains more uronic acid, sulphuric acid radicals and hexosamines than heparin and the acetyl radicals that heparin lacks.

#### MATERIAL

The series was collected from the Ophthalmic Department and Ophthalmic Out-Patient Department of the Central Hospital of Kuopio from the period December 1, 1959—October 31, 1960. It comprised 25 patients treated with heparinoid. The patients were unselected. Every patient showed either incipient or relatively far advanced degenerative changes in the retina and/or the choroid. All the patients were started with 80 mg of Ateroid (Star) per day. The therapeutic dose was 30 mg and the maintenance dose 20 mg per day. At the same time they received 9,000 international units of vitamin A, 0.21 g of vitamin E and 37.5 mg of Ronicol (Roche) per day. During the therapy cholesterol determinations (Pearson *et al.*) were made twice a week in the department and monthly at the out-patient department.

The patients' vision had been deteriorating for 3 months—2 years before the therapy was started. In addition to degenerative changes, the following diseases were diagnosed in the patients:

1. Incipient cataract ..... 8
2. Obstruction of the central vein .. 1
3. Occlusion of the central vein .... 1
4. Atrophy of the optic nerve ..... 1
5. Opacities in the vitreous body .... 2

The distribution of the material by age and sex is shown in Table 1.

TABLE 1  
DISTRIBUTION BY SEX AND AGE

| Sex | Years |       |       |       |     | Total |
|-----|-------|-------|-------|-------|-----|-------|
|     | 42—50 | 51—60 | 61—70 | 71—80 | 80— |       |
| F   | 1     | 1     | 2     | 9     | 2   | 15    |
| M   | 2     | —     | 5     | 2     | 1   | 10    |

Women accounted for 60 and men for 40 per cent of the patients. Eighty four per cent of the patients were over 61 years old. The number of patients in whom the only ophthalmic symptom diagnosed was macular degeneration was 12.

#### CHANGES IN VISION DURING THERAPY

The patients were divided into 2 groups. A-patients had macular degeneration only, B-patients had some other ophthalmic disease concomitantly. The results are given in Tables 2 and 3.

TABLE 2  
CHANGES IN THE VISION OF A-PATIENTS DURING THERAPY

| Vision   | At the Beginning of Therapy |     | After Therapy of: |     |     |     |          |     |
|----------|-----------------------------|-----|-------------------|-----|-----|-----|----------|-----|
|          |                             |     | 1                 |     | 2—3 |     | 6 Months |     |
|          | dx                          | sin | dx                | sin | dx  | sin | dx       | sin |
| <0.1—0.1 | 3                           | 3   | 2                 | 2   | 1   | 1   | 1        | 1   |
| 0.1—0.3  | 5                           | 3   | 4                 | 2   | 1   | 1   | 1        | —   |
| 0.4—0.5  | 2                           | 4   | 2                 | 2   | 5   | 2   | 4        | 3   |
| 0.6—     | 1                           | 2   | 3                 | 6   | 4   | 8   | 5        | 8   |

<sup>1</sup> One patient had a prosthesis in the right eye.

TABLE 3  
CHANGES IN THE VISION OF B-PATIENTS DURING THERAPY

| Vision   | At the Beginning of Therapy |     | After Therapy of: |     |     |     |          |     |
|----------|-----------------------------|-----|-------------------|-----|-----|-----|----------|-----|
|          |                             |     | 1                 |     | 2—3 |     | 6 Months |     |
|          | dx                          | sin | dx                | sin | dx  | sin | dx       | sin |
| <0.1—0.1 | 4                           | 3   | 4                 | 2   | 4   | 1   | 4        | 1   |
| 0.1—0.3  | 2                           | 3   | 2                 | 4   | 1   | 3   | 1        | 2   |
| 0.4—0.5  | 5                           | 3   | 2                 | 1   | 2   | 2   | —        | 2   |
| 0.6—     | 2                           | 4   | 5                 | 6   | 6   | 7   | 8        | 8   |

The emphasis in the tables moved down and to the right as the therapy continued, *i.e.* vision improved. For A-patients the vision in 14 eyes (61 per cent) at the beginning of therapy was 0.3 or poorer, while after 6 months' therapy this was the case in only 3 eyes (13 per cent). For B-patients the vision in 12 eyes (46 per cent) was 0.3 or poorer at the inception of therapy, after 6 months in 8 eyes (31 per cent). At the beginning of therapy the vision of A-patients was 0.6 or better in 3 eyes (13 per cent), after 6 months in 13 eyes (57 per cent). In B-patients the vision was

0.6 or better in 6 eyes (23 per cent) at the beginning of therapy. No differences were observed in the improvement of vision between A- and B-patients.

On admission to hospital the patients' cholesterin values ranged from 192 to 396 mg%. Mathematical treatment of the cholesterin values showed that the cholesterin value of A-patients at the beginning of therapy averaged  $289.7 \pm 14.1$  mg% and of B-patients  $298.1 \pm 16.0$  mg%. During therapy the cholesterin value dropped to  $268.2 \pm 17.2$  mg% in group A and to  $276.5 \pm 17.6$  mg% in group B. The differences between groups A and B did not differ significantly from one another either at the beginning or at the termination of therapy. The drop in the cholesterin value, 21.5 mg% for the A-patients and 21.6 mg% for B-patients, was of the same magnitude in both groups.

#### SUMMARY

The series comprised 25 patients with macular degeneration. Twelve of them had macular degeneration alone (A) and 13 had other ophthalmic symptoms concomitantly (B). In addition to heparinoids the patients were given vitamins A and E and Ronicol. The vision of the A-patients was 0.3 or poorer in 61 per cent of the cases at the initiation of therapy, in 13 per cent only after 6 months of therapy. The vision was 0.6 or better at the beginning of therapy in 13 per cent, after 6 months in 57 per cent. The vision of the B-patients was 0.3 or poorer at the beginning of therapy in 46 per cent, after 6 months in 27 per cent. It was 0.6 or better at the beginning of therapy in 23 per cent, after 6 months in 65 per cent. Such distinct improvements in vision suggest that heparinoids are worth further study.

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## PRODUCTION OF «ANTI-ANTIBODIES» IN RABBITS<sup>1</sup>

APPEARANCE IN RABBIT SERUM OF «ANTI-ANTIBODIES» REACTING  
WITH AUTOGENOUS AND ISOGENOUS ANTIBODY, FOLLOWING AUTO-  
STIMULATION WITH PROTEIN ANTIGENS

by

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Production of «anti-antibodies» in rabbits following iso-stimulation as well as auto-stimulation with particulate antigens has been reported by Dubiski (5). More detailed data, however, were given of only those sera which had been produced by means of iso-stimulation. The possibility that the properties of these sera were due to iso-precipitins was not ruled out.

In the present work, attempts were made to produce anti-antibodies by the use of well-defined soluble protein antigens. This could be achieved in some instances by means of stimulation of rabbits with egg albumin and human gamma globulin.

### MATERIAL AND METHODS

*Reagents.* — The egg albumin (Ea) was a commercial, five times crystallized product (lot 4911) of Pentex Inc., Kankakee, Ill. The human gamma globulin (HGG) and the bovine gamma globulin (BGG) were also obtained commercially, the former from AB. Kabi, Stockholm, as a 12 per cent solution, the latter (lot DC1473) from Armour Pharmaceutical Co., Eastbourne, England. The highly purified diphtheria toxoid (lot 31/c), containing 1800 Lf/ml, and the horse antitoxin, containing 1200 Lf/ml, were

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obtained through the courtesy of Dr. P. Halonen of Lääketehtas Orion, Pharmaceutical Manufacturers, Helsinki.

*Estimation of »Anti-Antibody» Activity by Use of Sensitized Sheep Cells.* — The technique described elsewhere (1,2) for the Waaler-Rose test was followed. All rabbit sera were first absorbed with normal sheep cells. If not otherwise stated,  $1/3 \times \text{MAD}$  of the rabbit anti-sheep cell amboceptor was used for sensitization of the sheep cells. In some instances the test was performed with only half of the usual volume of the reagents.

*Agglutination of Sensitized ORh Positive Cells.* — The method of Harboe and Lundewall (6) as described in a previous paper (2) was followed with the exceptions that the rabbit sera to be tested were inactivated and absorbed with ORh positive cells.

*Preparation of Specific Precipitates.* — The method described elsewhere (2) for preparation of diphtheria toxoid—antitoxin precipitates in slight antigen excess was followed, with minor modifications when applied to other antigen-antibody systems.

*Absorption with Specific Precipitates.* — The technique described in a previous paper (2) was followed. Packed precipitates, instead of precipitate suspensions, were used in all absorptions.

*Absorption with Sensitized Sheep Cells.* — An amount of rabbit anti-sheep cell amboceptor corresponding to  $2 \times \text{MAD}$ , was mixed with one milliliter of packed washed sheep cells. The mixture was kept for  $1\frac{1}{2}$  hours in an incubator at  $+37^{\circ}\text{C}$ . The sensitized cells were then washed three times with a large volume of saline. One milliliter of rabbit serum dilution 1:8 was mixed with the cells in the tube, which thereafter was kept overnight in the refrigerator. The supernatant serum dilution was then separated by centrifugation.

*Stimulation of Rabbits.* — The following terms suggested by Coombs (4) and Dubiski (5) for different varieties of stimulation are used in the present paper: a) Auto-stimulation, *i.e.*, stimulation with the individual's own autogenous antibody denaturated in serological reaction with the corresponding antigen, b) Iso-stimulation, *i.e.*, stimulation with denaturated antibody deriving from another individual of the same species, and c) Hetero-stimulation, *i.e.*, stimulation with denaturated antibody of a different animal species.

If not otherwise stated, a course of stimulation consisted of a series of three injections weekly, given intravenously at one day's intervals. All sera were inactivated by keeping them for 30 minutes at  $+56^{\circ}\text{C}$  in a water-bath, if not otherwise stated.

Most of the heart punctures performed in the present work were made following the method of Buthala and Mathews (3), using a restraint board constructed according to their instructions. We have found this method most satisfactory. After having gained some experience we have been able to draw repeatedly large bleedings from tens of rabbits without need of anesthesia and with no ill effects to the animals.

*Group 1.* Six rabbits (Nos. 165–170) were given alum precipitated Ea for five consecutive weeks. The first four injections contained 0.5 mg

of Ea, the second four injections 1.0 mg of Ea and so on, up to 2.0 mg of Ea contained in each of the last four injections of the course of stimulation. Thus, each rabbit received a total of 20 mg of Ea, distributed over 16 injections. One week after the last injection about 50 ml of blood was drawn from each rabbit by heart puncture (Bleeding 1). Of 12 ml of each rabbit serum, specific precipitates were then prepared by adding Ea in slight antigen excess. Following a rest period of two weeks each rabbit received a second course of sixteen injections, consisting on alternate days of autogenous Ea—anti-Ea precipitate or of the supernatant remaining after removal of the precipitate from the serum. Four injections of 1 mg of Ea followed, and three days after the last injection a second bleeding was collected by heart puncture (Bleeding 2). One week later a third course of stimulation was commenced. First, 12 injections of Ea—autogenous anti-Ea precipitate, obtained from 12 ml of serum as described above, were administered during four weeks. Immediately before the last injection an ear test bleeding was collected from each of the rabbits (Bleeding 3). One week later the stimulation was continued by giving 8 injections of supernatant and finally four injections of 1 mg of Ea. Three days later, *i.e.*, about six months after the stimulation of the rabbits had been started, about 50 ml of blood was drawn from each rabbit by heart puncture (Bleeding 4).

*Group 2.* Six rabbits (Nos. 181—186) were first given an intravenous injection of 1 mg of Ea. This was repeated three days later. Following an interval of two weeks the stimulation with Ea was continued for five additional weeks, as described above for the rabbits of group 1, until 16 injections and a total of 20 mg of Ea had been administered to each of the animals. Four days after the last injection about 50 ml of blood was drawn by heart puncture (Bleeding 1). Since «anti-antibody»-like activity was found in these bleedings from three rabbits (Nos. 182, 185, 186), the animals in this group were not subjected to any stimulation with Ea—autogenous anti-Ea precipitates or with supernatants. Following a rest period of one month, however, four injections of 1 mg of Ea were given to all six rabbits in group 2. One week after the last injection a second heart bleeding (Bleeding 2) was collected by heart puncture. The studies carried out with these bleedings will be reported separately.

*Group 3.* This group consisted of 18 rabbits, 12 of which had been immunized with different preparations containing normal HGG for the purpose of producing anti-human globulin sera. Of these 12 rabbits four (Nos. 138—141) had for prolonged periods received human serum containing complexes of diphtheria toxoid with non-precipitating antitoxin and subsequently commercial HGG. One of these rabbits (No. 141) was the only one of group 3 which produced «anti-antibodies». Four other rabbits (Nos. 171—174) had been stimulated with precipitate formed in normal human serum by ammonium sulphate at 40 per cent saturation. Four rabbits (Nos. 161—164), again, had received commercial HGG alone for several weeks. — Of the remaining six rabbits in group 3, two (Nos. 179, 180) had received a course of stimulation with BGG, whereas

four (Nos. 150—153) had been stimulated with washed Ea—pooled rabbit anti-Ea precipitates treated with rheumatoid serum containing large amounts of rheumatoid factor (RF).

The «anti-antibody» producer rabbit No. 141 and the three other rabbits of its group were stimulated according to the following schedule. First, they were given subcutaneously, with Freund's incomplete adjuvant, 0.2 ml of whole human serum containing ca. 50 units/ml of non-precipitating diphtheria antitoxin and an equivalent amount of toxoid. The stimulation was continued with a six weeks' course of 18 intravenous injections of this human serum without adjuvant. One week after the last injection an ear test bleeding was taken (Bleeding 1). Three weeks later, stimulation with large doses of commercial HGG was started. First, the animals received during one month one subcutaneous and ten intravenous injections, each of them consisting of 0.25 ml of the gamma globulin solution. Immediately before the sixth injection an ear test bleeding was taken (Bleeding 2). Three weeks after the last injection ca. 50 ml of blood was drawn from each rabbit by heart puncture (Bleeding 3). A rest period of two months followed, after which the animals were given a second course of gamma globulin exactly like that described above. Two weeks after the last injection the rabbits were bled by heart puncture and sacrificed (Bleeding 4). The investigations reported in the present paper were carried out with this bleeding. Thus, each of the four rabbits 138—141 had during a period of seven months received 2.5 ml of whole human serum containing toxoid—antitoxin complexes, and about 650 mg of commercial HGG.

#### RESULTS

Three groups of rabbits, a total of 30 animals, were studied. The first group, consisting of six rabbits (Nos. 165—170), was subjected to two varieties of auto-stimulation. First, the animals were stimulated with Ea alone, and secondly with washed Ea—autogenous anti-Ea precipitates and supernatants left after removal of the precipitate from the serum. As no anti-antibodies were found in the first bleedings of group 1, a second group of six rabbits (Nos. 181—186) was subjected to auto-stimulation with Ea alone. An «anti-antibody»-like effect appeared in the serum of three rabbits (Nos. 182, 185, 186).

A third group consisted of 18 rabbits, 10 of which (Nos. 171—174, 161—164, 179, 180) had been subjected to auto-stimulation alone. Four rabbits (Nos. 138—141) had been subjected to both auto-stimulation and hetero-stimulation and four (Nos. 150—153) to auto-stimulation, iso-stimulation and possibly to hetero-stimula-

tion (if the RF is an antibody). The serum of one of the 18 rabbits in the group was found to contain an «anti-antibody» effect. This particular rabbit (No. 141) belonged to those having received both auto-stimulation by means of human serum antigens and hetero-stimulation by means of diphtheria toxoid—human non-precipitating antitoxin complexes. An interesting observation of unknown significance (8) was that this rabbit serum, unlike the other 17 sera of the group, gave in immunoelectrophoresis against normal human serum three distinctly separate lines in the gamma region of the human serum. Another observation worth mentioning was that the sera of the four rabbits (Nos. 150—153) having received stimulation with Ea—pooled rabbit anti-Ea precipitates treated with RF apparently contained considerable amounts of iso-precipitins causing agglutination of sensitized sheep cells by these sera. This was suggested among others by an «all or none» pattern of reactivity of the four rabbit sera with a number of individual sensitizing amboceptors, the pattern varying from rabbit to rabbit in the group.

Thus, an «anti-antibody»-like effect, *i.e.*, a capacity to agglutinate sheep cells sensitized with rabbit amboceptor, was observed in the sera of eight (Nos. 182, 185, 186, 141, 150—153) of the 30 rabbits studied. As this effect of four sera (Nos. 150—153) produced by means of iso-stimulation most probably was due to iso-precipitins they were not studied further.

In order to select for further study the best possible amboceptors, nine individual rabbit anti-sheep cell amboceptors were tested for their ability to sensitize sheep cells to the action of the «anti-antibodies». As seen from table 1, the «anti-antibody» sera 185 and 186 seemed to accept as their reactant partner all of the nine amboceptors tested, whereas serum 141 reacted with three amboceptors only. Two amboceptors, 49 and 121, were selected for further study. The smallest dose of these two amboceptors capable of rendering sheep cells agglutinable by the «anti-antibody» serum 185 was about  $1/12 \times \text{MAD}$ .

Next, the effect of absorption with various antigen—antibody complexes on the «anti-antibody» activity was studied. The results are summarized in table 2.

It can be seen from table 2 that the «anti-antibody» activity could be removed by absorption with sensitized sheep cells and

TABLE 1

REACTIVITY OF «ANTI-ANTIBODY» SERA WITH A NUMBER OF INDIVIDUAL RABBIT  
ANTI-SHEEP CELL AMBOCEPTORS

| Amboceptor<br>No. | Agglutination<br>Titer of<br>Amboceptor | Agglutination Titers of «Anti-Antibody»<br>Sera for Sensitized Sheep Cells |           |           |
|-------------------|---|--|-----------|-----------|
|                   |   | Serum 141  | Serum 185 | Serum 186 |
| 48                | 4000                                    | <16  | 256       | 64        |
| 49                | 5600                                    | <16  | 1024      | 512       |
| 50                | 5600                                    | <16  | 128       | 64        |
| 51                | 4000                                    | <16  | 256       | 128       |
| 121 <sup>1</sup>  | 200                                     | 256  | 1024      | 512       |
| 125 <sup>1</sup>  | 800                                     | 64   | 512       | 128       |
| 134 <sup>1</sup>  | 400                                     | 128  | 256       | 128       |
| 187               | 560                                     | <16  | 256       | 128       |
| 188               | 200                                     | <16  | 64        | 64        |

<sup>1</sup> Rabbits stimulated for one year with sheep cells. A significant decrease in the agglutination titer had taken place during the long immunization period.

TABLE 2

ABSORPTION OF «ANTI-ANTIBODY» ACTIVITY WITH ANTIGEN-ANTIBODY COMPLEXES

| Complex Used<br>for Absorption                       | Agglutination Titers of «Anti-Antibody» Sera for<br>Sensitized Sheep Cells |                     |                        |                     |
|--|--|---------------------|------------------------|---------------------|
|  | Serum 141 <sup>1</sup>   |                     | Serum 185 <sup>2</sup> |                     |
|  | Before<br>Absorption   | After<br>Absorption | Before<br>Absorption   | After<br>Absorption |
| Sensitized sheep<br>cells                            | not tested   |                     | 1024                   | <16                 |
| Autogenous speci-<br>fic precipitate                 | 256  | <16                 | 1024                   | <16                 |
| Ea—isoegenous anti-<br>Ea precipitate                | 256  | <16                 | not tested             |                     |
| HGG—isoegenous<br>anti-HGG preci-<br>pitate          | not tested   |                     | 1024                   | <16                 |
| Diphtheria toxoid<br>—horse antitoxin<br>precipitate | 256  | 256                 | 1024                   | 1024                |

<sup>1</sup> Amboceptor 121 used for sensitization of the sheep cells.

<sup>2</sup> Amboceptors 49 and 121 used for sensitization of the sheep cells. Both gave identical results.

also with three different specific precipitates containing rabbit antibody, including that containing autogenous antibody. In contrast to this, the specific precipitate containing horse antibody did not exert any measurable effect on the «anti-antibody» activity of the two sera studied.

Experiments carried out with sera 185 and 186 showed that autogenous precipitates prepared within a range extending from an antibody excess of at least eight times that required for equivalence to an antigen excess of at least eight times that required for equivalence were capable of removing all measurable «anti-antibody» activity from the serum. In control experiments it was found that Ea and HGG alone, *i.e.*, without the presence of their corresponding rabbit antibody, did not inhibit the «anti-antibody» activity of sera 141 and 185, respectively.

The «anti-antibody» activity of sera 185 and 186 was also investigated using ORh positive cells sensitized with three different incomplete anti-D sera («Ripley», «Ta» and «Li»)<sup>1</sup>. No agglutination whatsoever was caused by the two «anti-antibody» sera.

In order to find out whether normal rabbit serum would inhibit the «anti-antibodies», the effect of eight normal rabbit sera upon the agglutination of sensitized sheep cells by 4—8 agglutinating doses of «anti-antibody» sera 185 and 186 was investigated. Seven of the sera did not exhibit any measurable inhibitory effect, whereas the eighth serum showed a slight effect, *i.e.*, inhibition in dilution 1 : 4 (1 : 16 when calculated from the total volume of the reagents).

#### DISCUSSION

By means of iso-precipitins, rabbits can be divided into at least seven allotypic serum groups (7). Thus, difficulties due to iso-precipitins are likely to arise when analysing the properties of «anti-antibody» sera produced by stimulation with antibodies of other individuals of the same species (iso-stimulation). Theoretically, these difficulties are avoided when stimulating the animal with autogenous antibodies (auto-stimulation). In practice, however, it is not easy to rule out entirely the possibility of contamina-

<sup>1</sup> The authors express their thanks to Drs. Marion Waller and John H. Vaughan for the serum «Ripley» and to Dr. H. R. Nevanlinna for the sera «Ta» and «Li».



tion of the needles and syringes by antigenic impurities, among them traces of serum of other individuals of the same species.

The most conclusive evidence ruling out the iso-precipitins is the absorption of the «anti-antibody» by autogenous precipitate. This was achieved in the present work. Additional evidence pointing in the same direction was offered by the inability of normal rabbit sera to inhibit the «anti-antibody» activity. In this respect the «anti-antibodies» found in the present work differed from those obtained by Dubiski by means of iso-stimulation (5).

The «anti-antibodies» found in four rabbit sera (Nos. 182, 185, 186, 141) fulfilled the criteria of true anti-antibodies so far as they apparently were directed against antibodies denaturated in serological reaction with the corresponding antigens. The quality of the antigen did not seem to be important, as rabbit antibodies of three different specificities studied were accepted by the «anti-antibodies» as reactant partners. The specificity of the «anti-antibodies» in the experiments described above, however, seemed to be limited to rabbit antibodies, and in one instance (No. 141) to some rabbit antibodies only. Horse diphtheria antitoxin of the flocculating variety and human incomplete anti-D antibodies did not show any reactivity with the «anti-antibodies» contained in the rabbit sera. It remains to be seen whether or not «anti-antibodies» that cross the «species barrier» can be produced by varying the time schedule of stimulation or the dosage or quality of antigen. Experiments along these lines are in progress.

The «anti-antibodies» described in the present work differ from immuno-conglutinins in not demanding for their action the presence of hemolytic complement (4, 9).

#### SUMMARY

«Anti-antibodies» causing agglutination of sheep cells sensitized with rabbit anti-sheep cell amboceptors were produced in rabbits by means of auto-stimulation with soluble protein antigens.

The «anti-antibodies» reacted with isogenous antibodies denaturated in serological reaction with the corresponding antigens. They also reacted with autogenous antibody and were not inhibited by normal rabbit sera. They did not react with flocculating horse diphtheria antitoxin or with human incomplete anti-D antibodies.



The «anti-antibodies» resisted heating for 30 minutes at  $+56^{\circ}\text{C}$ . They did not require the presence of hemolytic complement for their action.

*Note.* — After this paper had been submitted for publication, the February issue of «Arthritis and Rheumatism» was received, containing the Proceedings of the Seventh Interim Scientific Session of the American Rheumatism Association. In a paper presented at the session, Abruzzo and Christian described the production in rabbits, by means of auto-stimulation with *E. coli*, of a «rheumatoid factor-like substance» closely resembling the «anti-antibodies» described by us.

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## UTILIZATION AND STORAGE OF VITAMIN B<sub>12</sub> DURING PROLONGED FEEDING OF A GLUCURONIDOGENIC SUBSTANCE, CINCHOPHEN, TO RATS

by

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The measured B<sub>12</sub> activity in the tissues and serum may be taken as an indicator of the B<sub>12</sub>-vitamin status in the whole organism. In such clinical vitamin B<sub>12</sub>-deficiency conditions as in pernicious anemia, tape-worm anemia, postgastrectomy syndroms or during dietary deficiency one can usually find low serum values for this vitamin. Also the tissue contents diminish in B<sub>12</sub>-deficiency, but the reserves are assumed to be exhausted rather slowly. Low hepatic B<sub>12</sub>-activity has been found in the liver biopsies made for untreated pernicious anemia patients or in the liver of patients who have died from this disease (1, 24, 9, 2, 21, 20). The effect of dietary deficiency of B<sub>12</sub> on the tissue B<sub>12</sub> contents has also been studied by Jaffe (14). He analysed the liver and kidney contents of 10 generations of rats which were kept on a low B<sub>12</sub> diet. The tissue analyses revealed low figures. In the same investigation it was found that relatively short dietary deficiency periods were needed to decrease the B<sub>12</sub>-contents in the rat tissue. Already 3 weeks on the deficient diet lowered the B<sub>12</sub> levels in these tissues of previously undepleted rats to about 1/2 of normal values. Some other investigators were able to elevate the plasma B<sub>12</sub>-contents by carbon tetrachloride provoked hepatic injury (25). The liver B<sub>12</sub>-levels were not changed in this acute type of experimentation.

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It was assumed that the elevation in the blood B<sub>12</sub> was caused by a temporary release of this B<sub>12</sub> from the storage by the toxic agent. Such increase was not found in rats which were first kept 3 months on a B<sub>12</sub> deficient diet. The depletion of the storage was actually verified by liver analyses.

Vitamin B<sub>12</sub> deficiency in man is manifested clinically among other respects as a well known anemia. The effect of avitaminosis on the hematological picture is also studied in animal experiments (15, 22, 13). The results in animal experiments cannot be directly compared with human studies, because it seems obvious that in most animal species cyanocobalamin is not of the same importance in hematopoiesis than in man. The pig is nearest the man in this respect. Eg. in the anemia caused by B<sub>12</sub>-avitaminosis in the pig vitamin B<sub>12</sub> effects a strong reticulocytosis and remission. Despite of this the white blood count and bone marrow picture differ from the corresponding pictures in human anemias caused by the avitaminosis. The blood picture of the rat reacts on vitamin B<sub>12</sub> deficiency not similarly with the human. A deficient diet lowering the tissue levels did not affect the blood picture (12). Some other workers (21) made gastrectomies in rats with a lowered vitamin B<sub>12</sub> liver content and also an anemia as a result. The anemia did not react on vitamin B<sub>12</sub>, but was corrected with chlortetracyclin. The etiology of this anemia remained obscure. Many workers consider that a B<sub>12</sub>-deficiency does not greatly effect the hemopoietic process in the rat. In this respect one might feel that the rat is not the best possible object in these experiments, but according to the literature no animal species seems to react analogously with the human. Anyway tissue and serum vitamin B<sub>12</sub> levels in rat can very well be compared with the corresponding values in humans. Blood picture differs more of human.

In our previous studies by one of us (7, 8) it was found that cinchophen causes a rather specific reduction in the mucus secretion by the duodenal pyloric glands. We have also demonstrated a local gastrointestinal glucuronide conjugation function carried by the mucous membrane (3, 4). A concept of competitive functions by the mucous membrane has been suggested in light of these observations (5, 6). According to this hypothesis the function of the glucuronide conjugation system when loaded excessively might be carried on with the cost of other functions of the same cellular

elements. This would then explain the depression and even cessation of the mucous (mucoprotein) production.

The absorption and utilization of vitamin B<sub>12</sub> from the diet needs the action of intrinsic factor produced by the gastric and duodenal mucous membrane.

The present studies were undertaken in order to find if prolonged loading of the glucuronide conjugation system affects the utilization of dietary B<sub>12</sub>-vitamin.

#### MATERIAL AND METHODS

Altogether 60 albino rats (Wistar) were used of which 30 served as controls. Half of the animals in both groups were females. The animals were weaned at the age of 21 days and kept thereafter on a low B<sub>12</sub> diet until the end of the experiments. Water and diet food were given ad libidum.

The basic diet (23) was

Cornflour 65 weight units

Low vitamin content casein 28 weight units

Brewer's yeast 5 weight units

Salt mixture 2 weight units, according to (10), Fe content doubled.\*

Analyses of the B<sub>12</sub> content of this diet gave  $3.10^{-8}$  g/g.

The experiments were started when the rats had reached the age of 35—37 days. The weight curves are illustrated in fig. 1. The experimental group received daily a cinchophen suspension over a period of 8 weeks. The dosage is given in table 1. This suspension was made of a 0.5% tragacantha solution the cinchophen content of which was 25 g/1000 ml. The mixture was thoroughly shaken each time before the daily use. The weight of the animals were checked weekly (fig. 1). When the animals were sacrificed by intraperitoneal sodium pentothal injection the weight, blood hemoglobin, the erythrocyte, leucocyte and reticulocyte counts were taken together with the differential count and hematocrite determination. The B<sub>12</sub> contents were analysed from the serum, myocardium, gastric wall, kidney, adrenals and liver. Jaffe *et al.* (14) have stated that

\* The authors are greatly indebted to Messr. Lääke Oy for the disposal of this material for our studies.

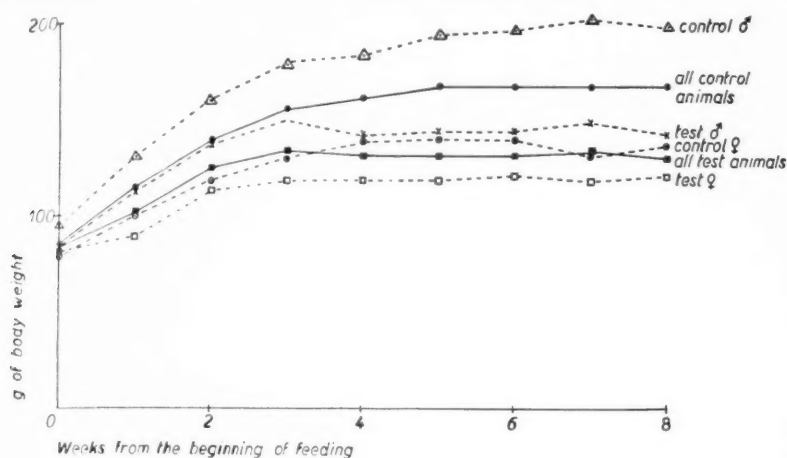


Fig. 1. — Body weight changes during the experiment.

TABLE 1

BLOOD PICTURES AFTER PROLONGED FEEDING OF CINCHOPHEN. THE DOSAGE OF CINCHOPHEN FOR THE TEST GROUP WAS 500 MG/DAY/KG. OF BODY WEIGHT DURING 1—4 WEEKS AND 300 MG/DAY/KG. OF BODY WEIGHT DURING 5—8 WEEKS

|                           | Test Group             |           |           | Control Group          |           |           |
|---------------------------|------------------------|-----------|-----------|------------------------|-----------|-----------|
|                           | All Animals            | ♂         | ♀         | All Animals            | ♂         | ♀         |
|                           | $\bar{X} \pm \epsilon$ | $\bar{X}$ | $\bar{X}$ | $\bar{X} \pm \epsilon$ | $\bar{X}$ | $\bar{X}$ |
| Hgb. g % . . . .          | 11.5 $\pm$ 0.9         | 13.5      | 9.8       | 14.6 $\pm$ 0.3         | 14.6      | 14.9      |
| RBC m/mm <sup>3</sup> . . | 5.37 $\pm$ 0.51        | 6.79      | 4.15      | 7.32 $\pm$ 0.15        | 7.10      | 7.51      |
| WBC/mm <sup>3</sup> . . . | 5715 $\pm$ 599         | 7217      | 4429      | 5718 $\pm$ 288         | 6150      | 5333      |
| MCH . . . . .             | 23 $\pm$ 1.2           | 20        | 25        | 20 $\pm$ 0.4           | 20        | 20        |
| Retic. % . . . .          | 1.1 $\pm$ 0.2          | 1.0       | 1.1       | 0.9 $\pm$ 0.2          | 0.7       | 1.1       |
| Hct % . . . . .           | 42 $\pm$ 2             | 42        | 43        | 44 $\pm$ 2             | 41        | 47        |
| Hypersegm. % .            | 27 $\pm$ 5             | 22        | 30        | 22 $\pm$ 3             | 19        | 25        |
| Ly % . . . . .            | 69 $\pm$ 5             | 73        | 66        | 74 $\pm$ 3             | 77        | 71        |
| Mon % . . . . .           | 3 $\pm$ 0.5            | 4         | 3         | 4 $\pm$ 0.4            | 4         | 3         |
| Eos % . . . . .           | 0.3                    | 0.4       | 0.3       | 0.8                    | 0.7       | 0.8       |
| Bas % . . . . .           | 0.0                    | 0.0       | 0.0       | 0.1                    | 0.1       | 0.1       |
| Segm. % . . . .           | 0.0                    | 0.0       | 0.0       | 0.1                    | 0.1       | 0.0       |

when using the proportional content of vitamin B<sub>12</sub> in organs (the total content of vitamin B<sub>12</sub> in liver and kidneys pro body weight) as a measure of dietary deficiency, the results are similar if the absolute amount of B<sub>12</sub> in tissue weight unit is used. The latter method is employed in this work.

**Vitamin B<sub>12</sub> Assay.** — The microbiological method which takes advantage of the specific dependency of the growth of *Euglena gracilis* var. *bacillaris* on availability of B<sub>12</sub> was used.

This method has originally been introduced by Hutner *et al.* (11). A description of the test is given by Ross (19). The basic nutritive solution was modified according to Nurmikko and Virtanen (18). The stock solutions were as follows:

|    |  |        |           |
|----|--|--------|-----------|
| A. | Fe SO <sub>4</sub> · 7 H <sub>2</sub> O .....  | 60.0   | mg        |
|    | Mn SO <sub>4</sub> · 4 H <sub>2</sub> O .....  | 24.6   | »         |
|    | Cu SO <sub>4</sub> · 5 H <sub>2</sub> O .....  | 0.26   | » /200 ml |
| B. | KH <sub>2</sub> PO <sub>4</sub> .....  | 500.0  | »         |
|    | Ca (NO <sub>3</sub> ) <sub>2</sub> · 4 H <sub>2</sub> O .....                              | 297.0  | »         |
|    | Mg SO <sub>4</sub> · 7 H <sub>2</sub> O .....  | 300.0  | »         |
|    | L-Glutamic acid .....  | 4000.0 | »         |
|    | DL-Malic acid .....  | 4000.0 | »         |
|    | Thiamine chloride .....  | 2.0    | » /400 ml |
| C. | CoCl <sub>2</sub> · 6 H <sub>2</sub> O .....   | 80.5   | »         |
|    | (NH <sub>4</sub> ) <sub>6</sub> Mo <sub>7</sub> O <sub>24</sub> · 4 H <sub>2</sub> O ..... | 36.8   | »         |
|    | Zn SO <sub>4</sub> · 7 H <sub>2</sub> O .....  | 44.0   | »         |
|    | NH <sub>4</sub> VO <sub>3</sub> .....  | 2.2    | » / 50 ml |

These solutions were used in a ratio A:B:C = 1: 4: 0.2. The incubation was made in regular test tubes in a thermostat room with constant temperature 27—29°C and constant illumination. The incubation period was 10 days. A 10 days old cellsuspension with optical density of 100—200 units in the Beckman DU spectrophotometer was used. The dilution of the incubation suspension was made with the basic nutritive mixture. The carry over of the vitamin is negligible with this method. The incubation was made by placing 1 drop of the suspension from a specially drawn pipette into each tube. The growth was measured with the spectrophotometer using the wavelength 660 mμ. Three samples were taken from each tube. Control series were run simultaneously with each test using a standard B<sub>12</sub>-series with increasing concentration. A commercial B<sub>12</sub> preparation was used as the stock solution and this was tested against the crystalline vitamin B<sub>12</sub>-substance 500612 of Merck AG.

**Treatment of the Samples.** — The serum samples were diluted 1: 40 and heated for 30 minutes at 100°C after they were added to the incubation mixture (19, 17, 16). The specimens were kept at —15°C if stored before the analyses.

The tissue specimens were first treated with papain at pH 5.0 (20). KCN activation and conversion in a concentration of 0.25 mg/ml was employed in the digestion. The samples were taken from 1 per cent homogenates and the incubation was carried out with 50 mg papain/g. fresh weight tissue 2 hours at 56°C.

## RESULTS AND DISCUSSION

The weight changes during the experiment are illustrated by fig. 1. Since it was attempted to provoke early as possible signs of B<sub>12</sub> deficiency the experiments were performed in growing rats. It was reasoned that the nucleic acid synthesis is more rapid during growth. The results indicate that cinchophen did not stop the growth but only retarded it. At the beginning of the experiment this effect is not yet present but is evident after 3 weeks.

The effect of the cinchophen treatment in the blood is listed in table 1. The animals developed anemia, in the female more clearly. No changes were found in the total amount of leucocytes. Neither were there any changes in the differential counts of them nor in the hematocrite values or reticulocyte counts. Abnormal types of cells were not found.

The values of the serum B<sub>12</sub> and tissue B<sub>12</sub> contents are listed in table 2.

TABLE 2

VITAMIN B<sub>12</sub> CONTENTS OF SERUM AND VARIOUS ORGANS AFTER PROLONGED FEEDING OF CINCHOPHEN (500 MG/DAY/KG. OF BODY WEIGHT DURING 4 WEEKS AND 300 MG/DAY/KG. OF BODY WEIGHT FOR 4 ADDITIONAL WEEKS). THE RESULTS ARE EXPRESSED AS 10<sup>-9</sup> G PER ML OF THE SERUM AND PER G OF FRESH WEIGHT OF THE TISSUE

| Group          | No.  | Serum         | Heart    | Stomach  | Kidney   | Adrenal  | Liver    |
|----------------|------|---------------|----------|----------|----------|----------|----------|
| Treated        |      |               |          |          |          |          |          |
| Male . . . . . | (15) | 496 (14)      | 366      | 297      | 624      | 266      | 378      |
| Female . . . . | (15) | 456 (12)      | 312      | 265      | 577      | 217      | 390      |
| Total . . . .  | (30) | 480 ± 23 (26) | 340 ± 24 | 282 ± 23 | 597 ± 20 | 242 ± 27 | 383 ± 13 |
| Control        |      |               |          |          |          |          |          |
| Male . . . . . | (15) | 488           | 280      | 255      | 595      | 287      | 329      |
| Female . . . . | (15) | 448           | 316      | 204      | 547      | 284      | 338      |
| Total . . . .  | (30) | 480 ± 13      | 297 ± 35 | 230 ± 22 | 576 ± 38 | 286 ± 28 | 333 ± 31 |

It can be seen that no significant difference exists in these values in control and cinchophen treated groups.

These result indicate that prolonged feeding of a glucuronidogenic substance, cinchophen, does not interfere with the utilization of dietary vitamin B<sub>12</sub>. It is known that rats do not developed

usual anemic changes in the blood constituents when kept in  $B_{12}$  deficiency even if the  $B_{12}$  levels are lowered in the blood or tissues.

The uptake of vitamin  $B_{12}$  from the food requires the intrinsic factor which has been shown to be present also in the rat gastric mucosa. The present studies may be taken as an indication that the formation and function of this factor obviously does not compete with the conjugation process of the mucosa.

#### SUMMARY

Prolonged feeding of a glucuronidogenic substance, cinchophen, to rats kept on a relatively low vitamin  $B_{12}$  diet, did not cause changes in the  $B_{12}$  contents of serum or tissues. These results would indicate that the mucosal conjugation processes do not interfere with the utilization of vitamin  $B_{12}$ . The conjugation processes demonstrated to be competitive to some other mucosal functions obviously do not compete with the production or function of the mucosal intrinsic factor.

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## STUDIES IN THE EFFECT OF INTRAUTERINE APPLICATION OF SORBIC ACID ON THE OESTROGEN INDUCED CHANGES IN RAT

by

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It has been shown that the hydrolysis of phenylglucuronide by  $\beta$ -glucuronidase was strongly inhibited by saccharic acid. In the same study it has also been shown that saccharic acid has no effect in the conjugation of o-aminophenol to a glucuronide (3, 4). The observation of the inhibitory effect of saccharic acid on the hydrolytic activity of  $\beta$ -glucuronidase has been confirmed (6, 10). All these studies have been made in vitro conditions. The same effect has in vivo also been studied. Karunairatnam and Levvy (4) could not find any effect of subcutaneously administered saccharate on the regeneration following liver injury or on the growth in mice.

By means of local intrauterine application we have been able to cause a partial reduction in the uterine  $\beta$ -glucuronidase activity (9). In these studies we have followed the changes caused by this inhibition on the oestrogen induced changes in the uterine size and nitrogen content (8). In the literature attention has been paid to the effect of sorbic acid (SoA), penta — 1:3 — diene-carboxylic acid, on  $\beta$ -glucuronidase activity. It has been claimed to have no effect in the  $\beta$ -glucuronidase activity in vitro (4) whereas in vivo such inhibitory effect has been found (5). In this study adult mice were given varying doses of SoA subcutaneously and the enzyme activity was analysed in the liver, kidney and uterus.

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A decrease was observed in the liver and kidney but not in the uterus. In growing mice the enzyme concentrations were reduced to the adult level. The rise in the enzyme activity following hepatic damage produced with carbon tetrachloride was not inhibited neither the rise following oestrogen treatment. On the other hand the usual rise after partial hepatectomy was inhibited. The authors speculate that the negative results obtained in part of the experiments might be due to inadequate dosage. SoA is quite a toxic compound and in order to produce a systemic effect nearly toxic amounts are needed. It was therefore considered to be worthwhile to study the effect of SoA in the oestrogen induced uterine changes by using the previously described local application procedure (9). By means of this intrauterine application it was expected to obtain high concentrations in this tissue without causing the generalized toxic effects. In our previous work the saccharate administration seemed to cause a potentiation of the estrogen effect indicated by changes in size and water content of the uterus (8).

#### METHODS

The experiments were made with 37 albino Wistar rats, which were ovariectomized bilaterally at the age of 3 months. The actual trials were performed 7 months after the operation. The animals were divided into 4 groups which are explained in table 1.

The experimental period was 7 days. On the 1<sup>st</sup> and 5<sup>th</sup> days oestradiolbenzoate dissolved in olive oil (Primogyn B oleosum, Schering A. G.-Leiras) in a dose of 100 ug per kg body weight was injected s. c. The daily local application of SoA introduced directly into the uterine cavity under light aether anesthesia was carried on between the 2<sup>nd</sup> and 6<sup>th</sup> day. The procedure has been described in our previous work. (9). The sorbic acid (Fluka) was dissolved in saline and the solution neutralized to pH 7 with sodium hydroxide. The solution was mixed into olive oil containing 4 per cent ethyl alcohol in order to improve the stability of the suspension. The final concentration of SoA was 120 mg per ml and the amount of the suspension applied each time was 0.2 ml per 100 g weight. Thus the total amount SoA given during the experiment was less than the single dose claimed in the previous studies to posses toxic effects (5). The control suspension was made accord-

TABLE 1

EFFECT OF INTRAUTERINE APPLICATION OF SORBIC ACID ON THE  $\beta$ -GLUCURONIDASE ACTIVITY OF UTERINE TISSUE, THE UTERINE SIZE, DRY WEIGHT/FRESH WEIGHT RATIO AND THE NITROGEN CONTENT OF THE UTERINE TISSUE IN OVARECTOMIZED RATS, EXPERIMENT PERIOD 7 DAYS, OESTRADIOL BENZOATE (100  $\mu$ G/KG) GIVEN S.C. ON THE 2nd AND 5th DAY

| No. of Animals | Oestrogen Treatment | Intra-Uterine Treatment | Mean Weight at Beginning | Mean Weight at End | Uterine $\beta$ -Glucuronidase Activity Units/g Fresh Wt. | Uterine Ratio $\bar{X} \pm \varepsilon$ | Dry Weight/Fresh Wt. $\bar{X} \pm \varepsilon$ | N-content mg/g of Tissue Wet Wt. $\bar{X} \pm \varepsilon$ |
|----------------|---------------------|-------------------------|--------------------------|--------------------|---|---|--|--|
| 5              | —                   | —                       | 259                      | 269                | 1136 $\pm$ 67   | 0.34 $\pm$ 0.04                         | 0.24 $\pm$ 0.008                               | 0.29 $\pm$ 0.01  |
| 5              | +                   | —                       | 246                      | 237                | 3257 $\pm$ 270  | 1.38 $\pm$ 0.18                         | 0.19 $\pm$ 0.008                               | 0.27 $\pm$ 0.03  |
| 13             | +                   | Control suspension      | 224                      | 201                | 3454 $\pm$ 122  | 1.39 $\pm$ 0.03                         | 0.20 $\pm$ 0.005                               | 0.26 $\pm$ 0.01  |
| 14             | +                   | Sorbic acid suspension  | 250                      | 216                | 3479 $\pm$ 115  | 2.00 $\pm$ 0.11                         | 0.17 $\pm$ 0.005                               | 0.28 $\pm$ 0.006   |

ingly but without the sorbic acid. The suspensions were always given heated to body temperature.

The animals were sacrificed on the 7<sup>th</sup> experimental day. Before this the weight was checked daily. After killing, the uterus was removed, wiped with filter paper and weighed. A 1 per cent homogenate was made using the Potter-Elvehjem glas homogenizer at the temperature of  $+7^{\circ}\text{C}$ . The  $\beta$ -glucuronidase analyses were made from 0.1 ml of the homogenate, duplicates from each sample. The enzyme activity was assayed according to the original method described by Talalay *et al* (11) and modified by Fishman *et al*. (2). In this procedure the incubation is carried at pH 4.50 in acetate buffer. Phenolphthalein-mono- $\beta$ -glucuronic acid (Sigma Chemical Co) was used as the substrate. The liberated phenolphthalein was measured at pH 10.45 (glycine-sodium hydroxide buffer). The nitrogen was estimated by the usual micro-Kjeldahl method. The ratio between the tissue dry and wet weight was obtained by weighing the tissue slice immediately after its removal from the body and after drying it over a period of 2 days at  $+90^{\circ}\text{C}$ .

#### RESULTS AND DISCUSSION

The results are listed in table 1. It appears that a similar decrease in the  $\beta$ -glucuronidase activity which was found in our previous experiments by using boiled potassium hydrogen saccharate (9) is not now present. Nevertheless the changes in the relative size of the uterus were similar to our previous finding. The relative uterine size is obtained from the ratio between the uterus weight and the body weight multiplied by thousand (7, 1). The oestrogen induced growth in the size of the uterus had increased in the SoA treated group as compared to the control group. The difference is significant as judged from the relative proportion of the dry weight. The water content is greater in the SoA treated group. This agrees with our previous experiments with saccharate. The nitrogen analyses did not reveal any marked differences. There is even an opposite tendency as in the dry weight fresh weight ratio. The difference, however, is not significant.

The lack of changes in the estimated  $\beta$ -glucuronidase activity in the uterine tissue when local application of SoA is used deserves some comments. In these experiments the oestrogen dosage is twice as great as in our previous experiments with saccharate.

This might be one reason for the unchanged  $\beta$ -glucuronidase activity. Oestrogen treatment as such increases this enzyme activity as can be seen also in our experiments. However, this does not suffice to explain why the enzyme activity is the same in the groups treated with the control suspension or the sorbic acid suspension. Apparently the in vitro measured  $\beta$ -glucuronidase activity does not always reflect the actual over-all effect of it in the tissues under varying conditions. Some earlier studies indicate also a difference even in the in vitro effects of sorbic acid and saccharate. Even though Karunairatnam and Levvy (4) have found that sorbic acid does not inhibit  $\beta$ -glucuronidase in vitro, Kerr *et al.* (5) were able to produce a reduction in the enzyme activity in the tissue, a cessation of growth and an inhibition of liver degeneration with this substance.

#### SUMMARY

Sorbic acid, a substance known to inhibit  $\beta$ -glucuronidase activity, was applied to ovariectomized rats by means of an intrauterine method. The enzyme activity, the relative size, and nitrogen content of the uterine muscle were registered. A decrease in the enzyme activity which was found in previous works using an other inhibitor, was not present. Nevertheless the changes in the relative size of the uterus were similar to the previous findings with another inhibiting substance saccharate. The possible explanations of this phenomenon are discussed.

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## 5-HYDROXYINDOLEACETIC ACID EXCRETION AFTER PNEUMONECTOMY

AN APPROACH TO THE PULMONARY DESTRUCTION OF 5-HYDROXY-  
TRYPTAMINE

by

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The lungs are rich in mono-amine oxidase capable of accomplishing the deamination of 5-hydroxytryptamine (5-HT). This circumstance and some experimental data on the destruction of 5-HT in perfused lungs (3), and the destructive action of lung extracts on 5-HT (6), are the main points on which the presumption of the pulmonary destruction of 5-HT is based. Further evidence is gained from observations in cases of the carcinoid syndrome. The endocardial lesions characteristic of this syndrome are predominantly right-sided. Since it is generally agreed that these lesions are caused in some way by circulating free 5-HT in excess, the most plausible explanation for the undamaged or less affected left-sided valves is a destruction of 5-HT during its lung passage.

We have tried to approach the problem indirectly by measuring the urinary excretion of 5-hydroxyindoleacetic acid (5-HIAA), the main metabolic product of 5-HT, after pneumonectomy.

### MATERIAL AND METHODS

The daily excretion of 5-HIAA was studied in seven patients undergoing pneumonectomy and in a reference group consisting of two cases of explorative thoracotomy and four cases of lobectomy. 5-HIAA was determined spectrophotometrically (8) on

two consecutive preoperative days and four times post-operatively after the immediate effects of the surgical procedure on 5-HT metabolism had passed off (2).

Because of their known actions on the 5-HIAA excretion, reserpine compounds, chlorpromazine and banana feeding were avoided.

#### RESULTS

The findings are shown in table 1. In most of the patients the 5-HIAA excretion was unaffected by the operation on the days in

TABLE 1  
5-HIAA EXCRETION BEFORE AND AFTER PNEUMONECTOMY

| Groups                        | Days before Operation |                | Days after Operation |                |                |                |
|-------------------------------|-----------------------|----------------|----------------------|----------------|----------------|----------------|
|                               | 2                     | 1              | 5                    | 6              | 9              | 10             |
| Pneumectomy                   | 3.7*<br>1.9—9.3       | 3.2<br>1.9—7.6 | 3.8<br>1.8—8.9       | 4.5<br>2.1—8.9 | 4.3<br>1.5—7.2 | 3.2<br>1.5—7.2 |
| Other thoracotomies . . . . . | 3.3<br>2.1—4.7        | 2.9<br>2.1—3.8 | 2.9<br>1.2—6.5       | 3.1<br>2.5—3.9 | 3.5<br>2.7—4.2 | 2.5<br>1.2—4.5 |

\* mean and range in mg/24 h.

question. In one case in the pneumectomy group and in two patients after other thoracotomies clearly low values were found after operation. Although the normal daily range of 5-HIAA excretion in man is rather wide, *i.e.*, 2—9 mg, the consecutive daily values in the same individual remain fairly constant (8). A variation of 20 per cent, however, must be taken into account. When the present findings are interpreted with this in mind, it is evident that no significant change occurred in either of the groups.

#### DISCUSSION

Although the daily 5-HIAA excretion is not a sensitive indicator of small variations in 5-HT decomposition, the above data suggest that lungs do not play a dominant role in 5-HT decomposition in normal human subjects. In cases of carcinoid syndrome the circumstances may be different. Normally a major part of 5-HT is located in the platelets and little if any is found in the plasma. In malignant metastatic carcinoid great amounts of 5-HT



is found in the blood and plasma and it is possible that a part of this free 5-HT is destroyed in the pulmonary circulation. Indeed, Goble *et al.* (4) were able to demonstrate a lower content of 5-HT in blood from the brachial artery than in blood drawn simultaneously from the pulmonary artery in a case of carcinoid syndrome. On the other hand, however, Sjoerdsma *et al.* (7) did not find a difference in the above respects in their patients.

With regard to the present experiments, the pulmonary hemodynamics after pneumonectomy may have had some significance. However, the alterations caused by pneumonectomy, well explored by animal experiments and studies on human subjects (1), are not in the direction that would enhance the enzymatic process in question.

As concluded from the present findings, no special role can be assigned to the lungs in the 5-HT breakdown process. This is in harmony with a statement from an authoritative source (5) that current evidence is not sufficient to allow lungs any unique ability to destroy 5-HT.

#### SUMMARY

The urinary excretion of 5-hydroxyindoleacetic acid was studied after pneumonectomy on the working hypothesis that if lungs would play a dominant role in the 5-hydroxytryptamine decomposition the removal of one lung would result in a decreased excretion of its main metabolic derivate. No decreasing trend was observed.

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INFLUENCE OF ROENTGEN IRRADIATION OF  
AUTOLOGOUS BLOOD IN VITRO ON MITOTIC RATE AND  
BLOOD CELL COUNT IN RATS<sup>1</sup>

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It has been reported earlier that fluctuations in mitotic activity have been obtained, by means of roentgen ray irradiation, not only in the area treated but also outside it (11, 12). The mitotic rate has increased or decreased according to the amount of irradiation used. Microscopically, too, similar distance effects have been obtained by roentgen rays: for example by treating one of two parabiotic rats with an epilation dose of roentgen to the skin, transient hair loss has been produced also in the corresponding area of the other rat (9). Teir (7) irradiated one external orbital gland of rats and found that there was an increase in mitotic rate after one day and a fall after one week not only in this gland but also in the other, nonirradiated gland. It can thus be concluded that radiation energy does not affect only the area treated: the effect is also reflected in different organs, especially homologous ones, in the form of fluctuations in mitotic activity.

Several factors have been put suggested as possible causes of these changes. They have been attributed for example to the necrohormones that may develop in connection with tissue degeneration of the treated area (1). The phenomenon is to be regarded as a distance effect of irradiation, which — according to Jolles (1)

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— might be transmitted humorally as action on molecules. Hematological factors also play a part, as indicated for instance by the tests of Friedrich-Fredska and Zaki (3): they injected blood of hepatectomized rats into normal rats and obtained a considerable rise in mitotic activity in the liver.

It is the purpose of this investigation to study whether agents of this kind are present in or released from the blood after *in vivo* high doses of roentgen, and whether these agents contribute to the fluctuations of mitoses at the periphery.

#### MATERIAL AND METHODS

Three-month-old white rats (Sprague—Dawley) were used for the experiments. They were all kept in a cage together. The animals were divided into five groups, each consisting of 5, with the exception of one group of 4 rats. They weighed from 165 to 190 gm. In each of the groups there were two females and three males, but in one two of each sex (in the group given 25,000 r all were females). One millilitre of blood was withdrawn from the left jugular vein of each rat in four groups under intraperitoneal nembutal anesthesia (0.1 ml.). To prevent coagulation, 0.1 ml. of 3.8% sterilized sodium citrate solution was added to the blood. This mixture was kept in a sterilized 2 ml. glass tube for 30 minutes. In three of the five groups the blood mixtures were irradiated, the doses being 5,000 r, 10,000 r and 25,000 r, and the duration of radiation respectively 10, 20 and 50 minutes. The following factors were used: 4 Al, 160 kV, and H.V.L. 0.4 mm. Cu. After irradiation the blood was reinjected into the right jugular vein of the same animals. The blood remained in the test tube for about 30 minutes in all groups. Two of the five groups served for control purposes. One of these was the so-called blood control group; blood samples were taken from each of the 4 rats in this group, stored for 30 minutes under the same conditions, and reinjected into the animals. The last group, consisting of 5 rats, was an ordinary control group.

The rats were killed two days after the experiments: their weights were recorded and biopsy specimens taken from the interscapular skin, the forestomach, glandular stomach, and the ileum. The specimens were fixed in Bouin's solution and the sections stained with hemalum eosin. Counts of mitoses were made, calcu-

lating separately the mitotic rates per 2,000 cells according to a method previously described (10). The statistical significance of the results was tested by Student's *t*-test (2).

In connection with decapitation, blood samples were also taken from the tails of each rat and red and white cell counts made, including differentiation of the granulocytes.

#### RESULTS AND CONCLUSIONS

The mitotic counts showed that there were no significant differences in the skin, forestomach and glandular stomach when comparing the mitotic rates in the blood control group and the irradiated groups with the mitotic rates obtained for the corresponding organs in the control group proper. The group treated with 10,000 r showed an exceptionally high mitotic rate in the skin ( $11 \pm 2.66$ ), but the difference was not significant: the mitotic rate in only one animal was high (25), the others were close to the control level. In the ileum the difference was almost significant ( $P < 0.05$ ) in the 10,000 r group and the blood control group, the mitotic rate being  $49.2 \pm 2.94$  and  $49.0 \pm 3.1$ , and the control value  $39.2 \pm 2.47$ .

In the group receiving 10,000 r there were signs of a possible response to treatment, and it was therefore decided to carry out still another experiment using the same roentgen dosage, rats of the same age, and the same experimental conditions. These rats were males, weighed 179–180 gm., and were divided into three groups. The control and the blood control groups each consisted of 5 rats and the experimental group proper of 8 animals. The samples were examined in the same way as before.

In the repeated experiment the mitotic rates for the organs concerned did not differ significantly in the different groups. In the forestomach only did an almost significant difference appear compared with the control group, when using 10,000 r: the mitotic activity showed an almost significant decrease ( $P < 0.05$ ).

In the original experiment, the white and red blood cell counts were normal and remained within the same limits as the control values; there were no statistically significant differences. Lymphocytes predominated and their level was equal in all groups, and the numbers of other granulocytes expressed as percentages did not show significant differences.

As far as the changes in blood counts in the second test are concerned, it may be noted that there were significant differences in the leukocyte counts in both the blood control group and the irradiated groups, but the erythrocyte count remained at the level of the controls in all groups. The differential counts showed very slight changes in the various granulocytes, and there were no significant differences. It was only in the group of neutrophils that a slight increase was observed in the first experiment in the groups treated with higher doses of roentgen. The lymphocyte counts were high in all groups and at the same level as in the control groups.

Both the experiments show that, under the experimental conditions here used, no major irradiation effect on the mitotic activity is produced with the doses used in ordinary roentgen ray therapy, or even with higher doses. In the first experiment, it is true, an almost significant difference was obtained in the mitotic rates for the epithelium of the ileum in the group of 10,000 r, but the effect must be accounted for the withdrawal of blood and reinjection, which is indicated by the fact that the same result was reached in the blood control group. In the second experiment the mitotic rate decreased almost significantly in the 10,000 r group whereas the same group showed a rise, though no significant one, in the first experiment.

Our experiments indicate that roentgen given to blood *in vitro* does not seem to release mitosis stimulating or inhibiting factors, not even when using higher doses. It has been demonstrated in previous experiments in tissue culture that irradiation of blood *in vivo* produces changes which are not found when blood plasma is irradiated outside the body and free from any contact with living cells. Using short wave irradiation transmitted by homogeneous telecobalt, Kivi (5) obtained marked prolongation of sprouting times of seeds with, for example, 12,000 r. Thus molecular changes are produced *in vitro* with roentgen doses that are higher than normal.

The blood changes caused by irradiation *in vitro*, do not seem to be of such a kind as would upset the mitotic equilibrium. This being the case, the blood changes must be due to irradiation *in vivo*, according to Spear (6) for example, the blood of an *in vivo* x-ray irradiated experimental animal causes changes in tissue culture

which are not produced with ordinary non-irradiated blood. If the blood plasma used in tissue culture is completely replaced by blood plasma of *in vivo* irradiated animals, this latter causes inhibition of tissue growth, while blood plasma irradiated outside the body has no inhibitory effect upon the growth of tissue explants suspended in culture media of which it is a constituent part.

To return to the changes in the blood count, the first experiment resulted in no appreciable fluctuations whereas in the later experiment the leukocyte counts were doubled as compared with the control values. This is obviously due to the withdrawal and reinjection of blood and the associated inflammatory factors; especially in the case of reinjection of blood this is possible. Teir, Rytömaa and Cederberg have (8) observed that removal of leukocytes through the intestine and their disintegration in the intestinal wall does affect the number of leukocytes in the blood. It is possible that at least a part of the white cells died in connection with the extravasal treatment and that factors affecting the number of white cells were thus liberated.

#### SUMMARY

1. One millilitre of blood was withdrawn from 3 months old rats divided into three groups, and the blood was irradiated *in vitro* with 5,000 r, 10,000 r and 25,000 r respectively (H.V.L. 0.4 mm. Cu). Two days after irradiation biopsy specimens were taken from the skin, forestomach, glandular stomach and ileum, and the mitoses were counted per 2,000 cells. Blood samples were also withdrawn in connection with decapitation, and counts of red and white blood cells were made, including differential counts of the latter. This series of experiments was repeated using a dose of 10,000 r.

2. The irradiation effect was fairly slight and no statistically significant difference was noted in the different groups, which shows that roentgen ray therapy administered *in vitro* to the blood releases neither stimulating nor inhibiting factors, not even when using fairly high dosage — in contradistinction to what has been reported earlier in the case of experiments *in vivo*. The leukocytosis must be interpreted as being due to inflammatory factors connected with the reinjection of blood into the animals.

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## EFFECT OF ETHYL ALCOHOL ON THE DEVELOPMENT OF EXPERIMENTALLY INDUCED ARTERIOSCLEROSIS IN RATS

by

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It has been found by numerous investigators that alcoholics less frequently suffer from arteriosclerosis than non-alcoholics (1—3), and it thus seems that alcohol retards the development of arteriosclerosis. Wilens (2) has attributed the differences between alcoholics and non-alcoholics to the relatively short life spans of the former and to their lower incidence of such factors as elevated blood pressure and diabetes, which promote arteriosclerosis. The studies of Dawber *et al.* (4), however, suggest that in man alcohol has only a slight influence on arteriosclerosis.

Only a few investigations have dealt with the significance of alcohol in the pathogenesis of arteriosclerosis. Eberhard (5) found that the serum cholesterol increased greatly in rabbits which were given alcohol, while Feller and Huff (6) found that alcohol does not alter the serum cholesterol level or influence atherogenesis. Gottlieb and co-workers (7) observed much higher serum cholesterol levels and more extensive vascular sudanophilia in young rats which had been given an atherogenetic diet and alcohol than in those which had received only the atherogenetic diet.

The object of the study reported in the present paper was to find out whether relatively small amounts of alcohol can modify experimentally-induced arteriosclerotic changes in rats.

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## MATERIAL AND METHODS

The animals were 40 female rats weighing from 185 to 210 grams. Their basic diet was modified by adding cholesterol and vitamin D<sub>3</sub> so that the dry food contained 2 per cent cholesterol and 25000 int. units of vitamin D<sub>3</sub> per 100 grams (8). The animals were divided into two equal groups, one group receiving only the diet mentioned and another receiving, in addition, 1 c.c. of 10 per cent alcohol twice daily through a cannula directly into the stomach. The animals were housed ten to a cage and ate and drank ad libitum.

After the animals had been on the diets for 5 to 8 months, two or three animals were removed from each group at intervals of three weeks or so and killed by decapitation. The heart, liver, kidneys and adrenals of each animal were removed and weighed, and specimens from these organs and the aorta were transferred to 10 per cent formalin. Microtome specimens were cut from the frozen aorta and stained with Sudan III. The degrees of sudanophilia and calcification were graded by one to four plus signs. The serum cholesterol levels of seven of the animals were determined by the method of Bloor and Sackett (9).

## RESULTS

The average weights of the two groups of animals and the average weights of various organs from the two groups are shown in Table 1. No significant differences were found between the average weights in the two groups, nor were any differences noted

TABLE 1

THE MEAN BODY WEIGHTS (AND STANDARD ERRORS) AND THE MEAN WEIGHTS OF VARIOUS INTERNAL ORGANS IN A GROUP OF RATS THAT WERE GIVEN AND IN A GROUP OF RATS THAT WERE NOT GIVEN ALCOHOL DURING PERIODS OF 5 TO 8 MONTHS. WEIGHTS IN GRAMS

|  | Body       | Heart | Kidney |       | Adrenal |       | Liver |
|--|------------|-------|--------|-------|---------|-------|-------|
|  |            |       | Left   | Right | Left    | Right |       |
| Group that was given alcohol .....     | 229 ± 4.37 | 0.83  | 1.00   | 0.96  | 29.0    | 26.3  | 8.32  |
| Group that was not given alcohol ..... | 236 ± 6.05 | 0.90  | 1.07   | 1.08  | 28.8    | 27.6  | 9.21  |

macroscopically in the internal organs or in the amounts of fat surrounding the various internal organs.

The distribution of macroscopic and microscopic changes in the aortas of the group which received and of the group which did not receive alcohol is shown in Table 2. A negative sign indicates

TABLE 2

MICROSCOPIC AND MACROSCOPIC CHANGES IN THE AORTAS OF THE RATS THAT RECEIVED AND IN THE AORTAS OF RATS THAT DID NOT RECEIVE ALCOHOL. — NO CHANGES; + SLIGHT MICROSCOPIC CHANGES; ++ DEFINITE MICROSCOPIC CHANGES, MAINLY IN THE MEDIA; +++ MACROSCOPIC GRANULES IN THE REGION OF THE AORTIC ARCH; ++++ AORTA TUBEROUS AND SCLEROSSED THROUGHOUT

|      | Alcohol | No Alcohol |
|------|---------|------------|
| —    | 10      | 4          |
| +    | 2       | 4          |
| ++   | —       | 3          |
| +++  | 3       | 4          |
| ++++ | 3       | 1          |

that no changes were seen in the aorta, one plus sign standing for slight microscopic changes, two plus signs for definite microscopic changes, mainly in the media, three plus signs for macroscopic granules in the region of the aortic arch, and four plus signs for an aorta which was tuberos and sclerosed throughout. The observed changes were no more frequent in the animals which had received alcohol for eight months than in those which had received it for five months.

No clear differences in the livers, kidneys, or hearts were noted in the two groups of animals. Slightly more lipids was observed in the suprarenal cortices of the animals that had received alcohol than in those that had not.

The mean serum cholesterol level in three rats which had not received alcohol was 141.6 mg/100 ml (range 139—146 mg/100 ml); the aortic changes in these rats were —, + and ++. In four animals which had received alcohol the mean cholesterol levels were 118.5 mg/100 ml (range 110—125 mg/100 ml) and the changes in the aorta —, —, +++ and ++++; the rat showing the greatest changes had also the highest cholesterol level.

Eight rats died during the experiment; two of these had been given alcohol. Among the surviving rats which had received alcohol there were three which were in a definitely poorer condition than any of the other surviving rats.

#### DISCUSSION

No changes were noted in the aortas of the majority of the rats that received alcohol. There was, however, a higher proportion that exhibited severe arteriosclerotic changes in this group than in the other group. These findings are in agreement with those of Feller and Huff (6) for rabbits and with those of Nicols and his associates (10) for chickens. The reason for the difference between our results and those of Gottlieb *et al.* (7) may have been the different diets employed. Gottlieb *et al.* believe that the atherogenetic effect of alcohol is partly due to an increased requirement of magnesium. The increased magnesium requirement caused by alcohol (11) may not become evident when the diet contains 20 per cent or more protein as in our experiments.

Alcohol did not greatly affect the serum cholesterol level, which it lowered rather than raised. The difference in the results obtained by others (5, 7) is due to the differences in the diet and to the greater amounts of alcohol administered.

#### SUMMARY

A study has been made of the effect of alcohol on the development of arteriosclerotic changes in the aorta induced by feeding cholesterol and vitamin D<sub>3</sub> to 40 female rats during periods varying from 5 to 8 months. The results suggest that alcohol has but little influence on the development of arteriosclerosis.

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## PROTEOLYTIC ACTIVITY OF HUMAN SERUM AND URINE AT VARIOUS pH's

by

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The proteolytic enzyme called pepsin has the pH-optimum 1.5–3 and that called cathepsin pH-optimum 3–5. The presence of these enzyme systems in the stomach, blood and urine of man is well known. In the determinations of the enzymes different authors have used different pH:s, *e.g.*: Merten, Ratzer and Kleffner (5) in the cathepsin determination of stomach pH 3.6–3.75; Buchs (2) in the cathepsin determination 1/25 N acetic acid solution and in the pepsin determination 1/25 N hydrochloric acid solution; The same solutions have been used by Sundberg (9) and Pfisterer (7); Gray, Ramsey and Reifenstein (3) in the uropepsin determination pH 1.5; Spiro, Ryan and Jones (8) in the plasma pepsin determination pH 2.3; Merten (4) in the uropepsin and urocathepsin determination pH 1.8 corr. 3.5; In the uropepsin determination of West, Ellis and Scott (12) the buffer solution is made acid to the methyl orange, *i.e.* pH 3 or less, by adding a sufficient amount of 2 N HCl; Baur (1) used in the urocathepsin determination pH 3.3 and in the uropepsin determination the method of West, Ellis and Scott (12). In his earlier investigations in the pepsin and cathepsin determinations the present writer (10, 11) has used the same pH:s as Baur (1) and West, Ellis and Scott (12). The following study was made in order to observe, how much the proteolytic activity of the same serum or urine sample varies with changing pH:s.

## MATERIAL AND METHOD

From 11 healthy persons and 9 patients with various gastrointestinal diseases and some anaemias the proteolytic activity of the same specimen of serum and urine was determined at pH 1.5, 3.0, 3.3, and 4.0. The blood sample was taken in the morning at 8.00 o'clock, and on the same morning the urine collection for a period of 24 hours was started. The proteolytic activity determinations were made with the method described earlier (10) edestin (Hoffmann-La Roche, Basel) being used as substrate. In the determinations the standard curves were drawn separately for each determination with the aid of the control tube containing 0.04 per cent of edestin and two other tubes with 0.03 and 0.02 per cent of edestin. The determinations were made in triplicates. The proteolytic activity of the serum was expressed in mg:s of edestin broken up in an hour by one ml. of the specimen to be studied, and the proteolytic activity of the urine was expressed in mg:s of edestin, which was broken up in an hour by the amount of urine excreted in an hour. The accuracy of the method was in the serum determination  $\pm 2 \times 0.15$  mg edestin/ml and in the urine determination  $\pm 2 \times 2.1$  mg edestin/hour, if the determinations were made in triplicates.

## RESULTS

The results appear from table 1. The individual values show great variability in different pH:s, and in many cases more than could be expected from the inaccuracy of the method. In the serum the greatest difference seemed to be between the values at pH 3.0 and 4.0 and in urine between those at pH 3.0 and 3.3. On an average, the serum values showed rising tendency at pH 3.3 and 4.0, but in the urine the highest values were at pH 1.5 and 3.0, especially at pH 3.0. Statistically the mean serum values at pH 3.0 and 4.0 differed significantly ( $P < 0.05$ ) in healthy persons. The urine mean values at pH 3.0 and 3.3 differed also significantly ( $P < 0.01$ ) in healthy persons. In various diseases the same tendency could be observed in the major part of the patients.

TABLE 1

PROTEOLYTIC ACTIVITY OF HUMAN SERUM AND URINE AT VARIOUS pH's

|                          |                      | Serum<br>Mg. Edestin/Ml |                 |        |                 | Urine<br>Mg. Edestin/Hour |                |                |        |
|--------------------------|----------------------|-------------------------|-----------------|--------|-----------------|---------------------------|----------------|----------------|--------|
|                          |                      | pH 1.5                  | pH 3.0          | pH 3.3 | pH 4.0          | pH 1.5                    | pH 3.0         | pH 3.3         | pH 4.0 |
| <i>Healthy subjects:</i> |                      |                         |                 |        |                 |                           |                |                |        |
| Age                      | Sex                  |                         |                 |        |                 |                           |                |                |        |
| 17                       | f                    | 0.85                    | 0.68            | 0.50   | 0.83            | 23.0                      | 60.8           | 3.0            | 31.3   |
| 21                       | "                    | 0.67                    | 0.15            | 0.52   | 0.00            | 1.7                       | 1.2            | 0.0            | 12.5   |
| 21                       | "                    | 0.30                    | 0.28            | 0.35   | 0.50            | 40.2                      | 50.6           | 10.1           | 12.0   |
| 45                       | "                    | 0.18                    | 0.15            | 0.39   | 0.40            | 38.1                      | 61.4           | 20.5           | 30.1   |
| 50                       | "                    | 0.37                    | 0.40            | 0.52   | 0.58            | 12.5                      | 28.4           | 19.0           | 20.1   |
| 25                       | "                    | 0.02                    | 0.00            | 0.17   | 0.19            | 20.6                      | 16.0           | 7.5            | 27.5   |
| 25                       | "                    | 0.15                    | 0.20            | 0.35   | 0.30            | 21.0                      | 30.0           | 12.5           | 10.0   |
| 70                       | "                    | 0.67                    | 0.45            | 1.00   | 1.25            | 10.2                      | 31.5           | 10.4           | 12.7   |
| 41                       | m                    | 0.37                    | 0.40            | 0.46   | 0.50            | 7.5                       | 21.0           | 27.5           | 25.0   |
| 65                       | "                    | 0.09                    | 0.45            | 0.95   | 1.04            | 19.2                      | 38.4           | 12.0           | 16.3   |
| 34                       | "                    | 0.15                    | 0.25            | 0.00   | 0.85            | 21.5                      | 15.0           | 12.2           | 7.8    |
| Mean                     |                      | 0.33                    | 0.31<br>± 0.057 | 0.47   | 0.58<br>± 0.115 | 19.6                      | 32.1<br>± 5.82 | 12.1<br>± 1.96 | 18.6   |
| <i>Diseases:</i>         |                      |                         |                 |        |                 |                           |                |                |        |
| Age                      | Sex                  |                         |                 |        |                 |                           |                |                |        |
| 77                       | f Pernicious anaemia | 0.13                    | 0.17            | 0.00   | 0.15            | 19.5                      | 9.0            | 4.0            | 10.5   |
| 71                       | " " " "              | 0.12                    | 0.30            | 0.35   | 0.00            | 6.8                       | 8.0            | 10.0           | 10.0   |
| 34                       | m Duodenal ulcer     | 0.18                    | 0.34            | 0.44   | 0.38            | 88.0                      | 115.5          | 54.0           | 16.2   |
| 46                       | " " " "              | 0.48                    | 0.35            | 0.78   | 0.56            | 91.0                      | 200.0          | 17.5           | 35.5   |
| 32                       | " Gastric ulcer      | 0.19                    | 0.40            | 0.57   | 1.22            | 18.2                      | 58.4           | 17.5           | 16.7   |
| 52                       | " " " "              | 0.37                    | 0.20            | 0.27   | 1.00            | 32.0                      | 48.1           | 20.0           | 22.5   |
| 70                       | " Gastric cancer     | 0.26                    | 0.90            | 0.30   | 0.41            | 15.6                      | 60.6           | 43.2           | 18.0   |
| 65                       | " " " "              | 0.35                    | 0.55            | 0.15   | 1.10            | 18.5                      | 22.5           | 16.0           | 0.0    |
| 42                       | " Acute pancreatitis | 0.28                    | 0.27            | 1.21   | 0.45            | 117.6                     | 111.3          | 31.5           | 27.7   |

## DISCUSSION

In the proteolytic activity of the serum significantly higher values were found at pH 4.0 than at pH 1.5 and 3.0, and in the urine significantly higher values at pH 3.0 than at pH 3.3 and 4.0 (fig. 1). This is in accordance with the results of Merten (4) who found in urine higher pepsin than cathepsin values. On an average, the uropepsin versus urocathepsin ratio in his material was 1.7-1.8, and in the present normal material it is 1.7. The finding that in the

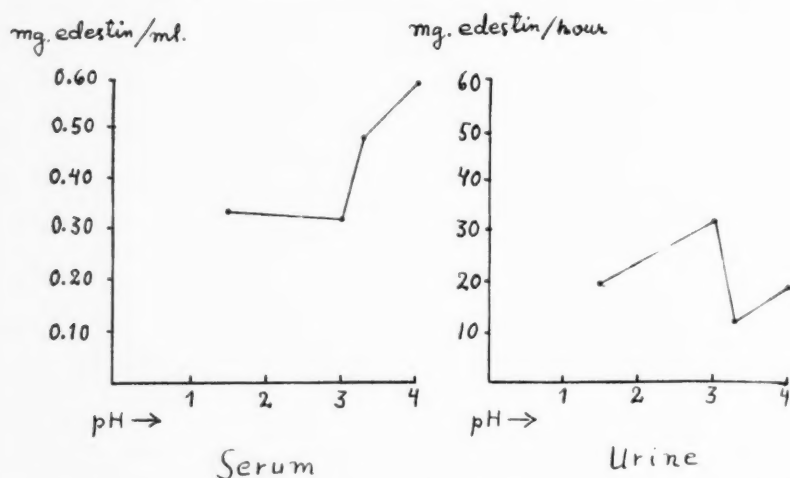


Fig. 1. — Mean proteolytic activity curves of serum and urine of 11 healthy subjects.

serum the values in the sphere of cathepsin are, on an average, higher than in the sphere of pepsin, is in accordance with the results of Mirsky *et al.* (6). The serum activity curve obtained in the present study (fig. 1) corresponds to that of the abovementioned authors. Merten, as well as Mirsky *et al.* used in their experiments hemoglobin-substrate.

Mirsky *et al.* (6) have found that plasma from healthy subjects contains at least three systems with proteolytic activity at acid reactions. One system, active at pH 3 to 4, is not dependent on the presence of the stomach and is inactivated at pH 1.5. A second system, which is also independent of stomach, is responsible for a minor fraction of the total proteolytic activity of plasma at pH 1.5 to 3 and is not affected by manipulations which inactivate pepsin. A third system, with maximum activity at pH 1.5 to 3, is responsible for the major part of the total activity of this reaction, and is derived from the stomach. The presence of these different enzyme systems in the plasma could thus account, at least partly, for the great variability of the proteolytic activities in the same subject at different pH:s. The greatest variability appeared between pH 3.0 and 3.3, which is considered to be the border of «pepsin» and «cathepsin».



## SUMMARY

Proteolytic activities were determined from the same sample of human serum and urine at pH 1.5, 3.0, 3.3, and 4.0. In the serum significantly higher values were obtained at pH 4.0 than at pH 1.5 and 3.0. In the urine significantly higher values were obtained at pH 3.0 than at pH 3.3 and 4.0.

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## DIFFERENCES IN DIHYDROSTREPTOMYCIN SENSITIVITY BETWEEN INDIVIDUAL *ESCHERICHIA COLI* BACILLI IN URINARY TRACT INFECTIONS

by

AULIKKI PITKÄNEN and TIMO KOSUNEN

(Received for publication February 15, 1961)

Urinary tract infections are either mixed infections — caused by several bacterial species — or are due to a single bacterial species. Even the latter, however, may be regarded as mixed infections if different strains of a single bacterial species are present. Much will depend on how the bacteria are differentiated. If elaborate methods are used the number of infections attributed to a single bacterial species will be reduced.

The present study deals with the differentiation of the *E. coli* isolated from one specimen of urine with the aid of a modified replica-plating method based on sensitivity differences towards dihydrostreptomycin (DSM).

### MATERIAL AND METHODS

*Urine Specimens.* — 41 clinical specimens containing DSM-sensitive *E. coli* were studied.

*Media.* — Ordinary agar plates and agar plates containing DSM in concentrations of 0.0, 0.05, 0.1, 0.2, 0.4, 0.5, 1.0, 2.0, 4.0, 8.0  $\gamma$ /ml of medium were used.

*Modified Replica-plating Method.* — A round steel stamp (Fig. 1) of 7.5 cm diameter, having 150 teeth was used. The length of a single tooth was 4 mm, the diameter 1 mm.

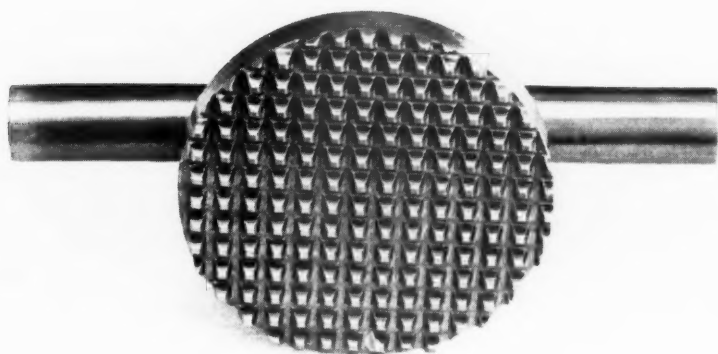


Fig. 1. — Steel stamp used in the modified replica-plating method.

Tenfold serial dilutions of the urine specimens were made in sterile saline. 0.1 ml of each dilution was spread on a dry agar plate which, after the fluid had dried, was incubated at 37°C for 16 hours. From a plate having separate colonies of 1 mm in diameter single colonies were transferred with a platinum loop, one colony to each tooth of the stamp. The stamp was then gently pressed on a series of agar plates containing increasing amounts of DSM. Three control plates with no DSM were included as the first, middlemost and last plate of the series. After an incubation period of 14 to 18 hours the colonies were recorded.

A study was made to check the differentiating ability of the method: Both DSM-resistant and DSM-sensitive «pure» coli strains — both also identifiable by their reactions with saccharose — were grown separately in broth. Equal amounts of DSM-resistant and DSM-sensitive coli broth cultures were combined and a series of tenfold dilutions was made. Each dilution was grown on ordinary agar plates. As a control each strain was also separately diluted and grown on agar plates. From plates with «mixed» strains, colonies were transferred to DSM-containing agar plates by the modified replica-plating method as explained above. Altogether ten pairs of different strains were used. As was expected, some of the colonies on the DSM plates either disappeared or showed weakened growth

when the DSM concentration was increased, whereas the growth of the rest of the colonies was not influenced by DSM. With the aid of the different saccharose reactions the results were easily checked and they were corresponding to the original reactions.

## RESULTS OF THE MAIN STUDY

In four cases of the 41 specimens of urine a sharp total inhibition of *E. coli* was seen when the DSM concentration was increased from 0.4 to 0.5γ/ml and in one case when it was increased from 1.0 to 2.0γ/ml. In 24 cases a gradual growth inhibition was seen, with smaller and smaller colonies, as the antibiotic concentration was increased. In these 29 cases all the colonies reacted similarly, however (homogeneous sensitivity). In table 1 an analysis is given of the 12 populations in which an irregular growth inhibition was seen («heterogeneous sensitivity»). In 4 of the 12 cases, *i.e.* Nos. 9, 21, 40, 44, we are inclined to consider the populations really

TABLE 1

THE NUMBER OF COLONIES GROWN FROM DIFFERENT SPECIMENS ON CONTROL PLATES AND ON PLATES CONTAINING VARIOUS CONCENTRATIONS OF DSM. THE SERIES OF PLATES ARE GIVEN IN ORDER OF INOCULATION. ONLY SPECIMENS WITH A «HETEROGENEOUS» SENSITIVITY ARE INCLUDED

| Specimen No. | Concentration of Dihydrostreptomycin |       |       |       |       |               |       |       |               |       |
|--------------|--------------------------------------|-------|-------|-------|-------|---------------|-------|-------|---------------|-------|
|              | 0                                    | 0.2   | 0.4   | 0.5   | 0     | 1.0           | 2.0   | 4.0   | 8.0           | 0     |
| 9            | 150 +                                | 129   | 122   | 112   | 150 + | 112           | 17    |       |               | 150 + |
| 10           | 150 +                                | 150 + | 150 + | 150 + | 150 + | 150 +         | 30    |       |               | 150 + |
| 12           | 150 +                                | 150 + | 150 + | 150 + | 150 + | 150 +         | 147   | 109   | 30            | 150 + |
| 13           | 150 +                                | 150 + | 150   | 150   | 150 + | 109           | 45    |       |               | 150 + |
| 14           | 150 +                                | 150 + | 150 + | 150 + | 150 + | { 71 +<br>79  | 21    |       |               | 150 + |
| 15           | 150 +                                | 150 + | 150   | 150   | 150 + | 143           |       |       |               | 150 + |
| 17           | 150 +                                | 150 + | 150 + | 150 + | 150 + | 150 +         | 150 + | 150 + | { 40 +<br>110 | 150 + |
| 21           | 150 +                                | 150 + | 150   | 150   | 150 + | 150           | 72    | 74    |               | 150 + |
| 29           | 150 +                                | 150 + | 150   | 150   | 150 + | 128           | 22    |       |               | 150 + |
| 30           | 150 +                                | 150 + | 150   | 150   | 150 + | 150           | 34    |       |               | 150 + |
| 40           | 150 +                                | 150 + | 150 + | 41    | 150 + | 27            | 4     | 2     |               | 150 + |
| 44           | 150 +                                | 150 + | 150 + | 150 + | 150 + | { 130 +<br>20 | 138   | 138   | 137           | 150 + |

+ = number of colonies with normal growth

± = number of colonies with inhibited growth

heterogeneous. In the remaining 8 a claim for heterogeneity remains doubtful in spite of a contrast to our homogeneous cases.

#### CORRELATION OF THE FINDINGS WITH THE DURATION OF THE CLINICAL INFECTION

When the results were compared with the duration of the clinical infection, it was seen that both in the acute and the chronic infections sensitivity could be heterogeneous.

#### DISCUSSION

The comparatively small number of colonies (150) investigated in this study gives no complete information as to the frequency of the populations with heterogeneous sensitivity. Obviously if a larger number of colonies had been examined or if more plates with interpolating concentrations of DSM had been used, more heterogeneous populations would have been found.

It is tempting to assume that in ascending urinary tract infections several non-identical bacteria could primarily invade the urinary tract simultaneously. Many of the cases enlisted in Table 1 could still be homogeneous but apparently not all. On the other hand even in the really heterogeneous cases it must be kept in mind that DSM sensitivity cannot be used as an absolute criterion of difference of strains since within a single strain, bacilli, obviously mutants, with different resistances have been found. (Silver & Kempe, 1947).

#### SUMMARY

The DSM sensitivity of individual colonies from urine specimens containing *E. coli* was studied by a modified replica-plating method.

Of the 41 specimens studied 29 showed quite homogeneous sensitivity. In 8 cases minor, and in 4 cases remarkable differences in DSM sensitivity between individual bacteria from the same specimen were observed.

Heterogeneous sensitivity was not correlated with the duration of the clinical infection.

## EFFECT OF SOME TUBULAR INHIBITING AGENTS ON THE URINARY OUTPUT OF SULPHATES

by

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(Received for publication March 10, 1961)

Sulphur is present in the organism both in inorganic and organic forms. The supply of the latter in the food is necessary since the organism is not able to synthesize organic sulphur from inorganic substances. The normal sulphate values in serum are: inorganic sulphate 2.4—3.3 mg, total sulphate 2.7—3.9 mg/100 ml. In the tissues sulphate is present as esters in sulphomucopolysaccharides, heparin, conjugated phenols etc.

Sulphur is excreted mainly in the urine although small amounts are eliminated with the feces as sulphides and from the skin and hair as keratin. The portion of the organic fraction of the excreted sulphur arrives from the protein catabolism so that the sulphur excretion varies with the amount of ingested protein and with the endogenous catabolism. Chemically 90 per cent of the sulphur excreted in the urine appears as inorganic sodium potassium or magnesium sulphate. Of the ethereal sulphates are the phenol, indoxyl and skatoxyl conjugates the most important. These components increase in intestinal putrefaction.

It has been previously shown that the inorganic sulphate in the plasma is completely ultrafilterable (1). Obviously all of the sulphate filtrated by the glomeruli is not, however, excreted into the urine since the sulphate clearance has shown to be smaller than

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<sup>1</sup> Supported by a grant from the Sigrid Jusélius Foundation.

glomerulus filtrate determined by endogenous creatinine clearance (2). In kidney diseases the endogenous sulphate clearance is only 40 per cent of the glomerulus filtration. (3) Also the indican clearance which reflects the clearance of an individual ethereal sulphate, is about the same order, 41.5 cc per minute (4). By means of intravenous administration of sodium sulphate it has been shown that the ratio between the sulphate concentrations of the urine and the plasma never reaches the corresponding ratio of creatinine so that tubular reabsorption must also be involved. (5).

All this evidence indicates tubular reabsorption. This may represent various forms. It can be entirely passive whereupon the substance diffuses freely through the cellular membrane. This is the case with urea. Active reabsorption may be of two different types, the other in which the amount of the absorbed substance depends only on the concentration so that even up to 90 per cent of the filtrate may be absorbed. This mechanism applies usually for salts. The active reabsorption may also be greatly limited which means that the capacity of the tubules is limited. This is the case when glucose, phosphate, uric acid and some vitamins are reabsorbed. It has been observed that sulphate is reabsorbed by an active type of mechanism which exhibits a limitation of reabsorptive capacity. Under condition of normal plasma sulphate and normal rates of glomerular filtration, there is quantitative reabsorption of all sulphate. With any slight increase in the tubulus load the reabsorptive capacity is rapidly exceeded and the excess is excreted in the urine (6). A theoretical reabsorption  $T_m$  curve for reabsorption of sulphate in the dog has been presented (7).

It has been shown in dogs that sulphate and thiosulphate mutually inhibited each others reabsorption in the tubules. For thiosulphate there is both reabsorption and secretion in the tubules and sulphates seem to depress both of them. On the other hand no secretion of sulphate has been able to be shown (8). Also glucose decreases the sulphate reabsorption  $T_m$  (9). The reabsorption obviously takes place in the proximal tubules since artificial damage of the kidney provoked by uranium takes place in the distal portion of the proximal tubule. The plasma sulphate level decreases simultaneously with an increased sulphate excretion in the urine (10).

According to the previous investigation there appears to occur

active transport in the tubules during the secretion of sulphates. The purpose of the present study was to find out what is the effect on the urinary sulphate excretion of substances known to exert a strong effect on the tubular functions, such as probenecid, acetazolamide, mercury diuretics and p-aminohippuric acid.

#### MATERIAL AND METHODS

Sixteen patients were given i.m. 0.14 mg a mercurial diuretic (Thiomerin R) and the daily urinary output of inorganic and total sulphate were followed and compared to the same during the control period without any treatment. 14 patients were given acetazolamide (Diamox R)  $0.5 \times 3$  and 15 patients received probenecid (Synergid R)  $0.5 \times 3$ . The daily changes in the urine were followed as above. 14 patients received p-aminohippuric acid in such amounts that the tubules were continuously saturated. Under these conditions the urinary output of inorganic and organic sulphate were followed over a period of 2 hours and these values compared to the same obtained on the preceding day. The patients taken for this study were chosen so that neither their history nor clinical studies revealed any kidney diseases and also that they suffered from no such disease — cardiac insufficiency anemia etc. — which causes disorder in the kidney functions. The diet of the patients was kept constant as regards to the protein content.

The urine  $\text{SO}_4$  determination was made with barium chloranilate. The total sulphate in urine was determined after acid and thermal hydrolysis. The accuracy of the method has elsewhere been described by Häkkinen *et al.* (11).

#### RESULTS

*Mercurial Diuretic.* — The biological actions of the mercuric ion and of organic mercurials have been ascribed to their ability to inhibit SH-activated enzyme systems. In the tubules they inhibit the effect of succinic dehydrogenase. They block the reabsorption of the chloride ions and diminish the potassium secretory transport. They have no effect on the phenolsulphonphthalein excretion (12).

During the control period the daily output of inorganic sulphate of the test subjects was  $0.44 \pm 0.07$  g and the total sulphate output



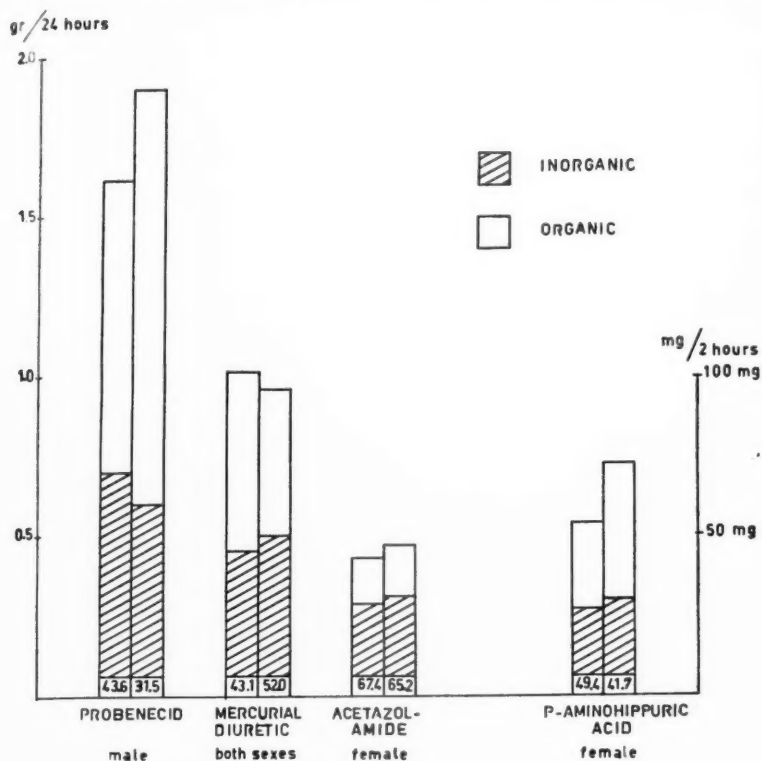


Fig. 1. — Effect of some tubular inhibiting agents on the urinary output of sulphates.

was  $1.02 \pm 0.12$  g. After giving the mercurial salts the corresponding figures were  $0.50 \pm 0.08$  and  $0.96 \pm 0.1$  g. No significant effect on the sulphate excretion could thus be noted.

*Acetazolamide.* — Acetazolamide inhibits the activity of carbonic anhydrase in the kidney also, thus causing an increased excretion of  $\text{HCO}_3^-$ ,  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{H}_2\text{O}$ . It also increases the phenolsulphonphthalein secretion so that it can by increasing the production of  $\text{H}^+$  ions in the tubular cells stimulate some secretory mechanisms (12).

During the control period the mean output of inorganic sulphate was  $0.29 \pm 0.04$  g and the total sulphate  $0.43 \pm 0.05$  g. During the acetazolamide treatment the corresponding means were  $0.31 \pm 0.04$  and  $0.48 \pm 0.06$  g. No significant effect could be detected.

*Probenecid.* — Probenecid inhibits the conjugation of glycine and benzoic acid. It has been shown that with an adequate dose probenecid completely inhibits the tubular secretory transport of penicillin. Also the renal transport of certain diagnostic agents such as diodrast and phenolsulphonphthalein is markedly depressed. It has been shown that carinamide, which resembles probenecid by its effects reduces the secretion of both thiosulphate and sulphate (13, 14).

During the control period the daily output of inorganic sulphate was  $0.70 \pm 0.08$  g and during the probenecid treatment  $0.60 \pm 0.07$  g. A decrease was noted in 9 cases, still the difference of the means is not significant. The means for the total sulphate were correspondingly  $1.61 \pm 0.21$  g and  $1.91 \pm 0.16$  g. An increase was present in 10 out of 15 cases.

When studying the output of organic sulphate it can be noted that prior to the treatment the daily secretion amounted to an average of  $0.91 \pm 0.09$  g and during the probenecid treatment to  $1.31 \pm 0.11$  g. Probenecid appears thus clearly to effect the organic sulphate secretion ( $t = 2.30$ ). It can also be noted that from the total sulphate secretion the portion of inorganic sulphate was markedly reduced from  $43.6 \pm 4.1$  to  $31.5 \pm 3.9\%$  ( $t = 2.20$ ) and of course correspondingly the organic compartment increases. Only in 2 individual cases out of 15 there was a slight decrease. Previous studies have shown that probenecid does not change the secretion of inorganic sulphate (13). The present study also indicates the same or that the tendency would rather be a reducing than increasing. On the other hand the matter is opposite in the case of organic sulphate. Since carinamide has been shown to reduce the secretion of inorganic sulphate, our own observation seems to indicate that the secretion of inorganic and organic sulphate is based on different mechanisms.

*P-amino Hippuric Acid (PAH).* — Part of the PAH excreted in the urine is filtrated through the glomeruli while the rest of it is actively secreted by the tubular cells. When the PAH contents in the plasma rises the secretory capacity of the tubuli cells for PAH is rapidly saturated and the part excreted by glomerular filtration increases. If diodrast or penicilline which also are secreted by the tubules are given simultaneously with PAH, the secretion of the latter is diminished due to the need of the same transport

capacity. Also a reduction of the opposite phenomenon, reabsorption, may take place. The reabsorption of ascorbic acid can be completely blocked temporarily by simultaneous administration of p-amino hippuric acid. Obviously the capacity of the energy for tubular cellular transport is the limiting factor.

During the control period  $24.7 \pm 3.8$  mg of inorganic and  $56.7 \pm 9.8$  mg total sulphate were excreted per two hours. During the PAH injection given on the following day the corresponding means for the same period were  $30.8 \pm 3.9$  mg and  $74.1 \pm 10.2$  mg. The differences were not significant.

The obvious sex differences shown in the total sulphate output presents as such no difficulties in the interpretation of the results since each patient served as its own control.

#### DISCUSSION

According to previous observations it has been noted that the sulphates are completely filtrated through the glomeruli but that incomplete reabsorption takes place in the tubules. For the sulphates the  $T_m$  appears to have a limited range.

By means of the applied procedures we have not been able to effect the reabsorption of the inorganic sulphates. Previously it has, however, been noted that carinamide diminishes it. On the other hand the excretion of organic sulphate was increased by giving probenecid.

Generally speaking probenecid diminishes the tubular secretion of foreign substances. Similar secretion of sulphate has not previously been shown to take place. One would be inclined to believe that the administration of mercury diuretics or PAH simultaneously secreted by the tubules would limit the secretory function and thus lead to a diminished sulphate secretion. Since the latter, however, is not the case, the secretory mechanism for sulphate appears to be implausible. Obviously probenecid in this case exerts its effect by diminishing the reabsorption of organic sulphate. This effect is similar with uric acid. Normally a high percentage of the uric acid filtered by the glomeruli is reabsorbed by the renal tubules and this is true even when plasma values are very much elevated. The transport system can be effectively

depressed by probenecid. We have been able to note that the reabsorption of inorganic and organic sulphate depends on different mechanism in the tubules. Similar dissociation of the excretory mechanism can be noted also for the 17-OHC-steroids. The hydrophobic free 17-OHCS are to 80—90 per cent reabsorbed in the tubules whereas the hydrophilic conjugated 17-OHCS are completely eliminated with the urine (15).

#### SUMMARY

Groups each consisting of 14—16 patients were given a mercurial diuretic, acetazolamide, probenic or p-aminohippuric acid. The output of inorganic and total sulphate in the urine was followed during the experimental and control period.

Only with probenecid could an effect on the sulphate excretion be noted. The inorganic sulphate excretion remained unchanged but the daily output of the organic sulphate was increased from  $0.91 \pm 0.09$  g to  $1.31 \pm 0.11$  g ( $t = 2.20$ ). The excretion of organic sulphates appears to take place similarly as the tubular transport of uric acid. The mechanism of the excretion of inorganic sulphate obviously depends on a different mechanism.

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## GROWTH, STABILITY AND HEMAGGLUTINATION OF A REOVIRUS<sup>1</sup>

by

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The reoviruses are a group of viruses recovered from human beings, chimpanzees, monkeys and calves (12). They are associated with the respiratory and enteric tracts, and serological studies in guinea pigs, rabbits, dogs and cats suggest that many other non-primates are naturally infected.

Many interesting biological properties of the reoviruses, such as a distinctive cytopathogenic effect in primate and many non-primate cell cultures (8), pathogenicity to newborn mice (1, 11), hemagglutinin to human type 0 erythrocytes (5), sensitivity of receptors to low periodate concentrations (2, 12), and multiplication in chick embryos (1), prompted a further study of the biological properties of these viruses.

This report describes the development of the infectivity, hemagglutinin and complement-fixing antigen titers of a reovirus in HeLa, amnion and human embryonic kidney cell cultures, thermal inactivation at various temperatures, factors affecting hemagglutination and hemagglutination inhibition, and the development of antihemagglutinating antibody in chicks immunized with reovirus.

### MATERIALS AND METHODS

*Virus.* — The »Lang» strain of type 1 reovirus was obtained from Dr. Leon Rosen, Laboratory of Infectious Diseases, National Institute of

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Allergy and Infectious Diseases, N.I.H., Bethesda, Md., U.S.A. In this laboratory it was passaged in amnion cell cultures and the stock of the third and fourth passage was used throughout the study.

*Cell Cultures.* — HeLa cells were grown in a medium containing 40% human serum in Hanks' solution. Human amnion cells of a continuous line obtained from Dr. R. Doorschodt, Hygienisch Laboratorium der Rijks Universiteit, Utrecht, Holland, were grown in a medium containing 25% calf serum in 0.05% lactalbumin hydrolysate medium. Human embryonic kidney cell cultures were prepared by the standard trypsinization methods from kidneys of 2—4 month old embryos. They were grown in a medium containing 20% calf serum in Eagle's minimum essential medium (3).

At the time of virus inoculation all cell cultures were washed 3 times with Hanks' solution before addition of the maintenance media. In HeLa and amnion cell cultures the maintenance medium was 5% chicken serum or 0.2% bovine albumin (Bovine Plasma Albumin Fraction V, Armour Pharmaceutical Co., Ltd., Eastbourne, England) and 5% tryptose phosphate broth in Eagle's minimum essential medium. In human embryonic kidney cell cultures the maintenance medium was 0.2% bovine albumin in medium no. 199. The volume of the medium was 1.0 ml in tubes, 7 ml in Carrel flasks and 60 ml in Roux bottles.

*Hemagglutination and Hemagglutination Inhibition.* — The hemagglutination methods used in this study are based on those of Rosen (10). Human type 0 erythrocytes were collected in Alsever's solution, washed 3 times in dextrose-gelatin-veronal solution, and stored as 10% suspension for 1—2 weeks. For the test, a 0.75% suspension of erythrocytes was made up with 0.85% NaCl solution containing 0.2% bovine albumin.

Hemagglutinin titrations were carried out in tubes or on plastic panels (13). To 0.4 ml of serial two-fold dilutions of virus, 0.2 ml of the 0.75% erythrocyte suspension was added. After thorough shaking, the tubes and plates were kept at room temperature for 2—3 hours. The sedimentation pattern was recorded and the endpoint was taken as reported by Rosen (10).

For the hemagglutination inhibition test, chick immune sera were treated with kaolin and human erythrocytes (10). To 0.2 ml of serial two-fold dilutions of serum 0.2 ml of hemagglutinin diluted to contain 4 units was added. The mixtures were kept at 36°C for 1 hour, unless otherwise indicated, before addition of 0.2 ml of erythrocyte suspension. The last serum dilution completely inhibiting agglutination was taken as the end point.

*Infectivity Titrations.* — In the preliminary experiments infectivity titrations were carried out in human embryonic kidney, human embryonic fibroblast, calf kidney, HeLa and amnion cell culture tubes. In each of these cell cultures the end point of titration was difficult to estimate by microscopic examination of the cytopathogenic effect, whereas the appearance of hemagglutinin in individual tubes gave highly reproducible results in infectivity titrations. The infectivity titers were highest when carried out in kidney cell culture tubes whether of human or calf origin. In human embryonic fibroblast cultures the titers were also high but near

the end point the appearance of hemagglutinin took as long as 3 weeks, as compared with 10–14 days in kidney cell culture tubes. In HeLa and amnion cell culture tubes the titers were 4–5 log units lower than in kidney cell culture tubes.

Hence in all the subsequent experiments the infectivity titrations were carried out by inoculating 4 human embryonic kidney cell culture tubes with 0.1 ml of each 10-fold dilution of virus. After 14 days' incubation at 36°C, the hemagglutinins in each tube were tested. The titers were calculated according to Reed and Muench (9) and expressed as log units of TCID<sub>50</sub>/ml.

*Immune Sera.* — Monkey immune serum (ECHO type 10) was obtained from the National Foundation, New York, N.Y., U.S.A. Guinea pig immune serum was prepared by immunizing the animals with fluorocarbon-treated reovirus grown in monkey kidney cell culture (6). Chick immune serum was prepared as indicated.

*Complement-fixation Test.* — The complement-fixation tests were performed by the standard technique used in this laboratory (7) employing two full units of complement and overnight fixation at 4°C. Monkey immune serum was inactivated for 30 minutes at 60°C, guinea pig immune serum for 30 minutes at 56°C. From each serum 8 antibody units were used in CF antigen titration. The two immune sera gave identical results. A control titration of complement in the presence of antigen was included in each test.

## RESULTS

### VIRUS GROWTH CURVES

The growth of reovirus was followed in HeLa, amnion and human embryonic kidney cell cultures in Carrel flasks which contained approximately 4, 5, and 5 million cells respectively at the time of virus inoculation. In each test 7–8 flasks of each type of cell culture were inoculated with 0.5 ml of undiluted virus with an infectivity titer of 6.7 (titrated in human embryonic kidney cell cultures). The cultures were incubated for 6 hours at 36°C and then washed twice with Hanks' solution to remove unadsorbed virus. After reincubation at 36°C one flask was taken at intervals (Figures 1, 2, and 3). 6 ml of the medium was collected, and the flask with 1 ml of medium and the cells was frozen at -25°C. After thawing, the medium with cells was collected and the flask washed with 1.0 ml of fresh medium which was pooled with the cell suspension. The pool was frozen and thawed twice at -60°C. Thereafter the volume of cell suspension was brought to 7 ml with fresh medium.



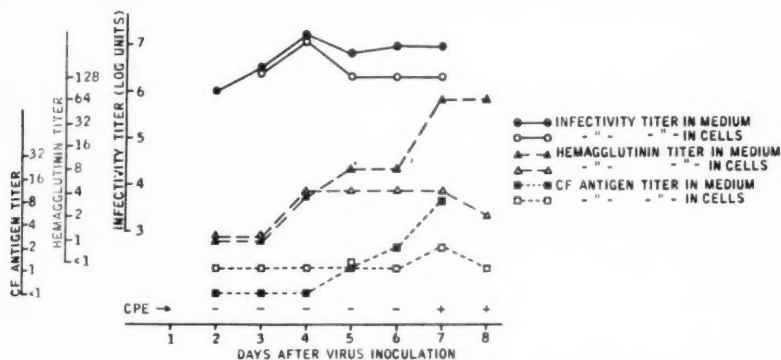


Fig. 1. — Development of reovirus infectivity, hemagglutinin and CF antigen titers and cytopathogenic effect in HeLa cell cultures.

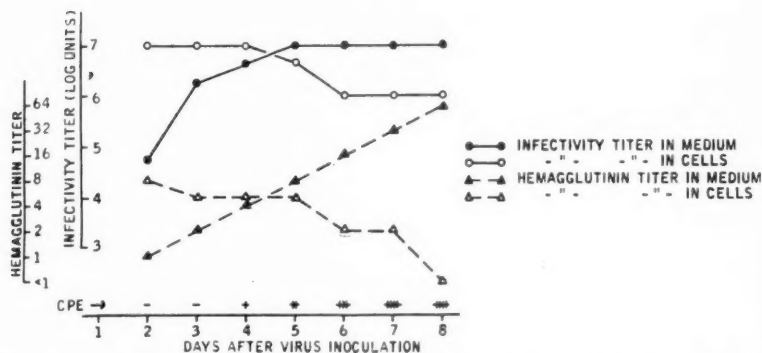


Fig. 2. — Development of reovirus infectivity and hemagglutinin titers and cytopathogenic effect in amnion cell cultures.

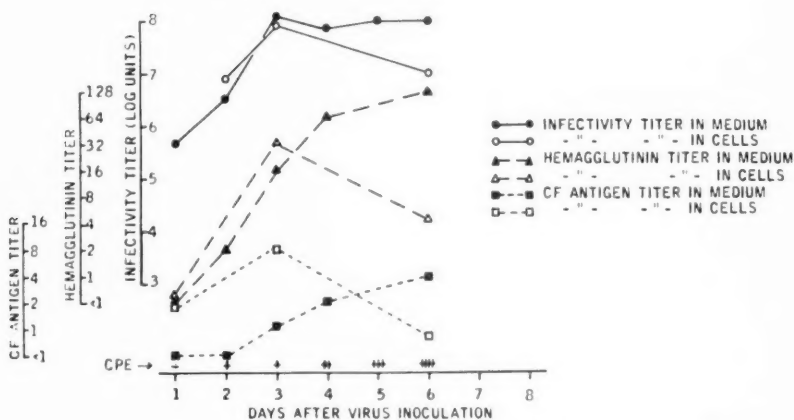


Fig. 3. — Development of reovirus infectivity, hemagglutinin and CF antigen titers and cytopathogenic effect in human embryonic kidney cell cultures.

The original medium and the medium with cells of each harvest were stored at  $-25^{\circ}\text{C}$  until the infectivity, hemagglutinin and CF antigen titrations were carried out. The results of the tests are given in Figures 1, 2, and 3, which also show the course of development of the cytopathogenic effect of reovirus in each cell culture system.

The infectivity titers reached the maximal level in the media of HeLa, amnion, and human embryonic kidney cell cultures in 4, 5, and 3 days respectively. Cell degeneration due to the virus could not be detected or it was in an early stage at that time. The highest infectivity titers (about 8 log units) were obtained in kidney cell cultures. The titers remained at a high level for the test period. By freezing and thawing of amnion cell suspensions the maximal infectivity titers were obtained even in the first specimen taken 2 days after virus inoculation.

In HeLa and human embryonic kidney cell cultures the hemagglutinin titers increased at about the same rate in cells and in medium during the first 3—4 days after virus inoculation. Later the titers in the cells levelled off and began to decrease, whereas in the medium the hemagglutinin titers continued to increase during the remaining 3—4 days of the experiment. In the frozen and thawed amnion cells the hemagglutinin titer, like the infectivity titer, reached the highest level in the first specimen taken. In the amnion cell medium, the hemagglutinin titer was 1:1 in the first specimen and increased two-fold each day of the test period. In other similar experiments it was found that no rise in hemagglutinin titer occurred after 8 days' incubation.

The development of hemagglutinin in two types of amnion cell media was compared. One medium contained 5% chicken serum and the other 0.2% bovine albumin. The rise of hemagglutinin titer was steeper in the albumin medium but the final titer was the same in both media 6 days after virus inoculation.

The development of CF antigenicity was tested in HeLa and human embryonic kidney cell cultures. In HeLa cells the CF antigenicity was present in low titers in the first specimen taken two days after virus inoculation and remained so throughout the experiment. In the medium of HeLa cells CF antigenicity appeared in 5 days and increased until at least the seventh day, no later specimen being available for the CF antigenicity test. In the human

embryonic kidney cell cultures the first cell specimen for CF antigenicity was taken one day after virus inoculation and the CF antigen titer was 1: 2. In 3 days it was 1: 8 and in 6 days had dropped below the detectable level. In the kidney cell medium the first specimen showed CF activity in 3 days and thereafter the titer gradually increased.

#### VIRUS STABILITY

For the virus stability tests, several lots of reovirus grown in amnion cell cultures, both in bovine albumin medium and in chicken serum medium, were centrifuged 10 minutes at 2000 r.p.m. The supernatant was collected and distributed into small tubes with rubber stoppers. The pH of the medium was adjusted to 7.2.

*Infectivity.* — The thermal stability of reovirus infectivity was measured at 36.5°, 56°, and 60°C. At 36.5°C inactivation was very slow (Figure 4). In three days no measurable decrease in the infectivity titer was detected. In three weeks the decrease was 2 log units. At 56°C the decrease in the titers in the experiment shown in Figure 4 was approximately 2 log units in 30 minutes and 4 log

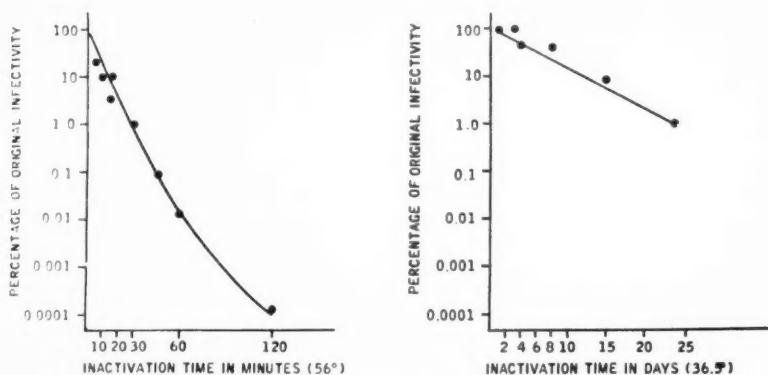


Fig. 4. — Inactivation of reovirus at 36.5°C and 56°C at pH 7.2.

units in 60 minutes but detectable virus remained at 120 minutes. In another experiment at the same temperature with a virus of 1 log unit lower infectivity titer, no virus was detected after 60 minutes. At 60°C about 4 log units of virus was inactivated in 5 minutes but thereafter the inactivation rate decreased; in 30 minutes infective virus was still detectable.

*Hemagglutinin.* — At 36.5°C no drop in hemagglutinin titer was observed in 23 days. At 56°C and 60°C inactivation was rapid; in 45 minutes and 5 minutes respectively no hemagglutinin activity remained (Figure 5). In some other experiments at 56°C a «tailing off» effect was obtained at the level of 1:4 and 1:2.

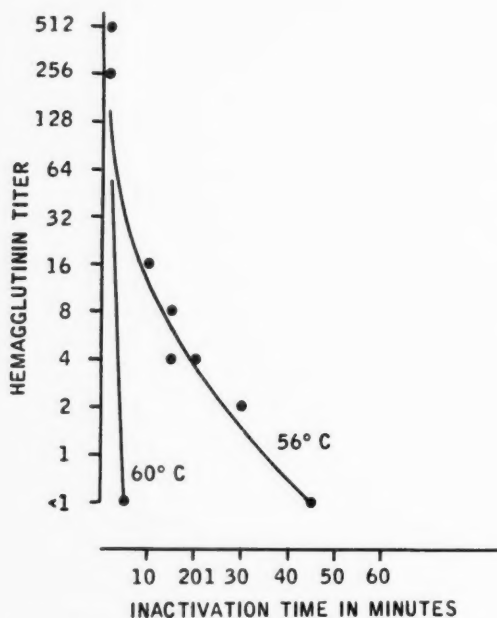


Fig. 5. — Inactivation of reovirus hemagglutinin at 56°C and 60°C at pH 7.2.

#### FACTORS AFFECTING HEMAGGLUTINATION AND HEMAGGLUTINATION INHIBITION

No effect of pH levels from 6.0 to 8.0, of erythrocyte concentrations from 0.5% to 1.5%, or of temperatures of 4°, 23°, and 36°C was observed on the reovirus hemagglutination and hemagglutination inhibition titers. On the other hand, the incubation time and temperature of mixtures of virus and chick immune serum dilutions before the addition of erythrocyte suspension was found to have a significant effect on the hemagglutination inhibition titer. In the experiment shown in Table 1 the hemagglutination inhibition titers were 4 times higher after overnight incubation than after 30 to 60 minutes at 23°C.

TABLE 1

THE EFFECT ON THE HEMAGGLUTINATION INHIBITION TITER OF THE INCUBATION TIME AND TEMPERATURE OF REOVIRUS AND CHICK IMMUNE SERUM MIXTURES BEFORE ADDITION OF ERYTHROCYTE SUSPENSION.

| Incubation Time and Temperature of Virus-Serum Mixtures | Dilution of Immune Serum |      |      |       |       |       |        |        |
|---|--------------------------|------|------|-------|-------|-------|--------|--------|
|   | 1/20                     | 1/40 | 1/80 | 1/160 | 1/320 | 1/640 | 1/1280 | 1/2560 |
| without incubation                                      | —                        | —    | —    | —     | +     | +     | +      | +      |
| 30 minutes at 23°C                                      | —                        | —    | —    | —     | ±     | +     | +      | +      |
| 1 hour at 23°C ..                                       | —                        | —    | —    | —     | ±     | +     | +      | +      |
| 2 hours at 23°C ..                                      | —                        | —    | —    | —     | —     | +     | +      | +      |
| 6 hours at 23°C ..                                      | —                        | —    | —    | —     | —     | —     | +      | +      |
| 1 hour at 36°C ..                                       | —                        | —    | —    | —     | —     | +     | +      | +      |
| overnight at 4°C ..                                     | —                        | —    | —    | —     | —     | —     | +      | +      |
| overnight at 23°C                                       | —                        | —    | —    | —     | —     | —     | ±      | +      |

— = no hemagglutination

± = partial "

+

#### DEVELOPMENT OF ANTIHEMAGGLUTINATING ANTIBODY IN CHICKS IMMUNIZED WITH REOVIRUS

A group of 2 month old chicks were immunized twice with 0.5 ml of reovirus given intramuscularly at an interval of 14 days. A blood specimen was taken at intervals (Fig. 6). In the pooled sera of 4 chicks the anti-hemagglutinating antibody titer was 1:40 7 days after the primary immunization of the chicks. After the second immunization the peak (1:640) was reached in 7 days, *i.e.* 21 days after the first immunization.

#### DISCUSSION

In the experiments reported it was found that the development of infective reovirus and specific viral antigens can be accurately studied in many cell culture systems. This with the notable thermal stability of the infectivity at 36.5°C, makes reoviruses very suitable agents for many types of theoretical studies, *e.g.* the possible role of the viral antigens developing in infected cells as precursors for mature virus. Such studies have hitherto been done mainly with myxoviruses. The development of infective virus in high titers without degeneration of cells in the early stage of infection in cul-

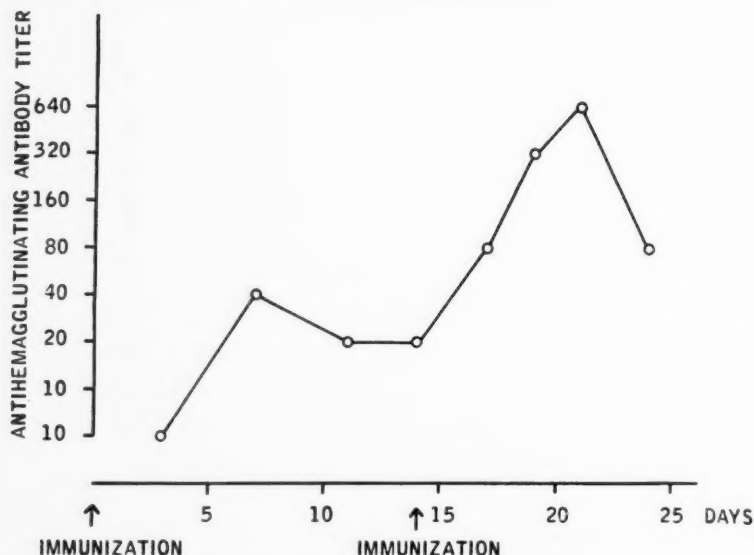


Fig. 6. — Development of antihemagglutinating antibody titer in chicks immunized by two injections (0.5 ml each) of reovirus. Each circle represents pooled sera of 5 chicks.

tured cells also makes this a suitable system for studying the nutritional requirements for the propagation of virus in cultured cells and for biochemical studies on host-virus interactions.

The preliminary data of Goldfield *et al.* (6) indicated that ECHO hemagglutinins (including ECHO type 10) were sedimentable by ultracentrifugation and were removed from the supernatant during the process of agglutination parallel with the infectious virus. In the present study a constant rise in hemagglutinin titer for 3—4 days after the infectivity titer had levelled off was observed in each of the three cell culture systems, but most clearly in the amnion cells. Unfortunately, the infectivity titrations were not carried out by such a sensitive method as was desirable, but even these results indicate that at least part of the reovirus hemagglutinin may be not associated with infectious virus. This was further emphasized by the thermal inactivation experiments at 36.5°C, where no decrease in hemagglutinin activity was observed in three weeks but the infectivity titers dropped 2 log units. On the other hand reovirus hemagglutinin was rapidly inactivated at 56°C where influenza hemagglutinin remains almost intact (4).

In the virus growth curves the development of the infectivity,

hemagglutinin and complement-fixing antigen titers in cells represent more the titers in cells and medium together, since a part of the original medium was left in Carrel flasks when cells were frozen and thawed. That is why the titers in «cells» did not decrease more during the last days of the experiments.

#### SUMMARY

It was shown that the development of reovirus infectivity, hemagglutinin and complement-fixing antigen titers can be accurately studied in HeLa, amnion and human embryonic kidney cell cultures. The maximal infectivity titers (up to 8.0 log units/ml) were observed 3—5 days after virus inoculation when the cytopathogenic effect of the virus could not be noted in the cells or was at an early stage. After the infectivity titers had levelled off, the hemagglutinin titers continued to rise for a further 3—4 days. In the frozen and thawed amnion cells high infectivity and hemagglutinin activity were observed two days after virus inoculation.

At 36.5°C the infectivity titer decreased 2 log units in three weeks, whereas no drop in hemagglutinin titer was observed. At 56°C the decrease in the infectivity titer was approximately 2 log units in 30 minutes; the hemagglutinin activity was completely lost in 45 minutes.

Erythrocyte concentrations of 0.5% to 1.5%, pH levels of 6.0 to 8.0, and temperatures of 4°, 23°, and 36°C had no effect on reovirus hemagglutination and hemagglutination inhibition titer. On the other hand, the incubation time and temperature of virus and chick immune serum mixtures before the addition of erythrocyte suspension had a significant effect on the hemagglutination inhibition titer.

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## COMPLEMENT-FIXATION TESTS FOR ANTISERA PREPARED AGAINST EPITHELIAL AND FIBROBLAST CELL STRAINS<sup>1</sup>

by

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(Received for publication March 15, 1961)

The complement-fixation reactions between HeLa strain and five cell strains derived from human breast carcinomas and rabbit immune sera prepared against these strains were described in a previous paper (2). No complement-fixing antibodies could be shown after cross absorption of the immune sera with the homologous or heterologous cell strains. These studies indicated that the HeLa cell strain and the five cell strains isolated from breast carcinomas by the authors have common antigens. By employing fractions of the immune sera separated by electrophoresis, it was established that the antibodies that reacted in the complement-fixation reactions were mainly  $\gamma$ -globulins (1). We have now studied various cell strains derived from different (human) tissues to determine their ability to bind the complement in immune sera.

### METHODS

The cells employed in the study were E.J cells (isolated from a breast carcinoma), HeLa cells, Chang's conjunctiva cells, and IJ and OB fibroblast cells isolated from Dupuytren's contractures. The cell strains were maintained in Roux flasks containing a mixture of 50 per cent human serum, 48 per cent Hanks' medium

<sup>1</sup> Presented at the XII Northern Congress of Pathology and Microbiology in Gothenburg, 1959.

and 2 per cent chicken embryo extract or of 10 per cent human serum, 88 per cent Eagle's solution and 2 per cent chicken embryo extract.

The preparation of immune sera, performance of complement-fixation tests and absorption technique were described in a previous paper (2).

#### RESULTS

The results of the tests are shown in Tables 1, 2 and 3. From Table 1 it is seen that the EJ, HeLa and Chang immune sera gave positive complement-fixation tests with all the corresponding cell strains as antigens with titres that were of the same magnitude. On the other hand, the IJ and OB immune sera prepared with the fibroblast cell strains gave negative complement-fixation tests with the EJ, HeLa and Chang strains but a positive test with the homologous fibroblast cell strain. These results indicate that the cell strains from cancerous tissues and Chang's conjunctiva cells, which were all of ectodermal origin, have common antigens. The fibroblast cell strains, which were of mesodermal origin, differed from the former strains by giving a positive complement fixation reaction only with the homologous strains. The same is evident from Tables 2 and 3 which show the results of cross-absorption tests. The homologous strains absorbed antibodies from immune sera. Antibodies in sera prepared with cell strains of ectodermal origin could be bound with these strains only. The fibroblast

TABLE 1  
RABBIT IMMUNE SERUM COMPLEMENT-FIXATION TITRES<sup>1</sup> FOR EJ (Breast carcinoma), HELA (Portio carcinoma), CHANG'S CONJUNCTIVA, AND IJ AND OB FIBROBLAST (Dupuytren's contracture) CELL STRAINS

| Rabbit Immune Serum | Titrated with Strain |          |          |     |         | Serum Control |
|---------------------|----------------------|----------|----------|-----|---------|---------------|
|                     | EJ                   | HeLa     | Chang    | IJ  | OB      |               |
| EJ 122              | 320-1280             | 320-640  | 320-640  | <10 | <10     | —             |
| HeLa 99             | 160-320              | 320-1280 | 320-640  | <10 | <10     | —             |
| Chang 175           | 320-640              | 320-640  | 320-1280 | <10 | <10     | —             |
| IJ 200              | 10-20                | <10      | <10      | 80  | 160-320 | —             |
| OB 262              | <10                  | <10      | <10      |     | 80      | —             |

Symbol: — = Complete lysis

<sup>1</sup> The complement-fixing antibody titres are numerically reciprocals of the serum dilution prior the addition of the complement, antigen and haemolytic system.

TABLE 2

ABSORPTION AND COMPLEMENT-FIXATION TESTS<sup>1</sup> ON RABBIT ANTISERA AGAINST EJ (Breast carcinoma), HELA (Portio carcinoma), CHANG'S CONJUNCTIVA, AND IJ AND OB FIBROBLAST (Dupuytren's contracture) CELL STRAINS

| Rabbit Immune Serum | Absorbed with Strain | Titrated with Strain |      |       |     | Serum Control |
|---------------------|----------------------|----------------------|------|-------|-----|---------------|
|                     |                      | EJ                   | HeLa | Chang | OB  |               |
| EJ 122              |                      | 320                  | 320  | 320   | 10  | —             |
|                     | EJ                   | <10                  | <10  | <10   | <10 | —             |
|                     | HeLa                 | <10                  | <10  | <10   | 10  | 10            |
|                     | Chang                | <10                  | <10  | 10    | 10  | —             |
|                     | OB                   | 160                  | 320  | 320   | <10 | —             |
| HeLa 99             |                      | 320                  | 320  | 640   | 20  | —             |
|                     | EJ                   | 20                   | 20   | 10    | 20  | 20            |
|                     | HeLa                 | 20                   | 20   | 20    | 20  | 20            |
|                     | Chang                | 20                   | 20   | 20    | 20  | 10            |
|                     | OB                   | 80                   | 80   | 80    | <10 | —             |
| Chang 175           |                      | 320                  | 320  | 320   | <10 | —             |
|                     | EJ                   | <10                  | <10  | <10   | 10  | —             |
|                     | HeLa                 | 10                   | 10   | 10    | 10  | 10            |
|                     | Chang                | 10                   | 10   | 10    | 10  | 10            |
|                     | OB                   | 160                  | 160  | 160   | <10 | —             |
| IJ 200              |                      | <10                  | <10  | <10   | 160 | —             |
|                     | EJ                   | <10                  | <10  | <10   | 40  | —             |
|                     | HeLa                 | <10                  | <10  | <10   | 40  | —             |
|                     | Chang                | <10                  | <10  | <10   | 40  | —             |
|                     | OB                   | <10                  | <10  | <10   | <10 | —             |

<sup>1</sup> For explanation see footnote to Table 1.

TABLE 3

ABSORPTION AND COMPLEMENT-FIXATION TESTS<sup>1</sup> ON RABBIT ANTISERA AGAINST TWO FIBROBLAST STRAINS DERIVED FROM DUPUYTREN'S CONTRACTURES<sup>1</sup>

| Rabbit Immune Serum | Absorbed with Strain | Titrated with Strain |      |       |     |     | Serum Control |
|---------------------|----------------------|----------------------|------|-------|-----|-----|---------------|
|                     |                      | EJ                   | HeLa | Chang | JP  | OB  |               |
| IJ 200              |                      | <10                  | <10  | <10   |     | 160 | —             |
|                     | EJ                   | <10                  | <10  | <10   |     | 40  | —             |
|                     | HeLa                 | <10                  | <10  | <10   |     | 40  | —             |
|                     | Chang                | <10                  | <10  | <10   |     | 40  | —             |
|                     | OB                   | <10                  | <10  | <10   |     | <10 | —             |
| JP 244              |                      | <10                  | <10  | <10   | 80  |     | 10            |
|                     | EJ                   | 10                   | 10   | 10    | 40  |     | —             |
|                     | HeLa                 | 10                   | 10   | 10    | 40  |     | —             |
|                     | Chang                | <10                  | <10  | <10   | 40  |     | —             |
|                     | JP                   | <10                  | <10  | <10   | <10 |     | —             |

<sup>1</sup> For explanation see footnote to Table 1.

strains were able to bind the complement of only the immune sera prepared with fibroblast cells.

The above results indicate that tissue culture cells of ectodermal origin derived from both cancer and normal conjunctiva tissues behave similarly in complement-fixation tests, but that fibroblast cells of mesodermal origin differ from the preceding cell types.

#### SUMMARY

Complement-fixation tests have been carried out using rabbit antisera prepared against three types of human cell strains, namely E.J and HeLa cell strains derived from breast and portio carcinomas, Chang's conjunctiva cell strain, and IJ and OB fibroblast cell strains derived from Dupuytren's contractures. Conforming complement-fixation titres 1/160—1/1280 were recorded for antisera prepared against E.J, HeLa, and Chang's conjunctiva cell strains when titrated with any of these strains whereas no binding of the complement by fibroblast strains was observed. Antisera prepared against fibroblast strains reacted only with the strains derived from Dupuytren's contractures. Hence, no cross-reaction between strains of ectodermal and mesodermal origin could be demonstrated by complement-fixation tests.

*Acknowledgements.* — This investigation was aided by a grant from the Sigrid Jusélius Foundation.

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## «DIRECT» REACTION OF ENZYMIC DIGEST OF HYALURONATE WITH *p*-DIMETHYLAMINO BENZALDEHYDE

by

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(Received for publication March 17, 1961)

The enzymic digests of hyaluronate may give a positive colour reaction with *p*-dimethylaminobenzaldehyde (1, 2). The colour vanishes by the action of air and sometimes a bluish transient tinge is noted. The chromogen substance is formed only when the hydrolysis is carried out at pH near the neutral.

Our preliminary experiments showed that the colour originates partly from the enzyme preparation, presumably from tryptophan, partly from the hyaluronate itself. To elucidate the contribution by the enzyme, partial hydrolyzates were prepared from electrophoretically purified hyaluronidase. As a comparison, lysozyme was subjected to similar treatment, since it has a high content of tryptophan and its enzymatic action resembles that of hyaluronidase. It was thought that the tryptophan-containing peptides may or may not be similar. It was found, incidentally, that the testicular hyaluronidase contained appreciably iron. For the study of the degraded hyaluronate, the digest was fractionated by ion exchange column (3). An Ehrlich-positive fraction could be isolated, but not characterized.

### EXPERIMENTS AND RESULTS

*Materials.* — The following enzymes were purchased: bovine testis hyaluronidase (Sigma, H 107—52), crystallized lysozyme (Sigma, L 78—77), salt free chymotrypsin (General Biochemicals Inc., Lot 30256) and papain (1:350, E. Merck, Lot 57967). The hyaluronate was prepared from um-



Later the solution tends to become opalescent. To 90 ml. of aqueous solution of the purified hyaluronidase 1 ml. of activated papain solution and 5 ml. acetate buffer (pH 5.5, 0.2 *M*) were added. Lysozyme solution (1%) was treated analogously and both were kept at + 37°C. for 48 hours.

When hyaluronidase solution was treated with  $H_2S$  to render it more suitable for papain action, an abundant iron sulphide precipitate was formed. The manufacturer informed us that no heavy metals had been used in the preparation of the hyaluronidase. Histochemical attempts to locate iron in the electrophoretic fractions were not successful.

The enzymatic digests were dialyzed three times against twenty-fold volume of distilled water at +2°C. The fluid inside the bag did not give any reaction with Ehrlich's reagent and was therefore discarded. The outer fluids were pooled and evaporated on a water bath down to 25 ml.

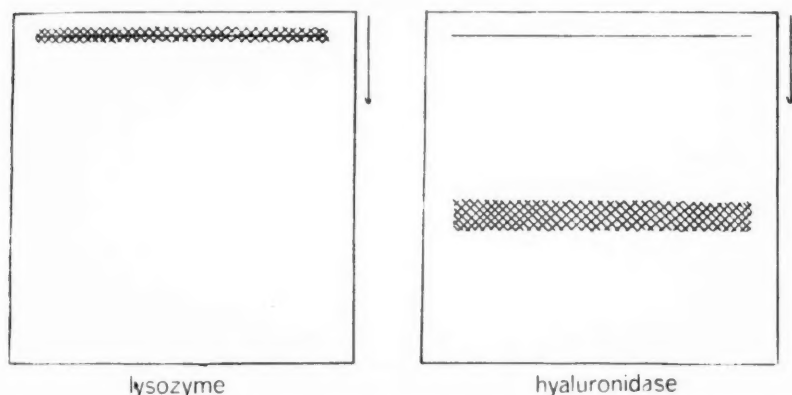


Fig. 2. — Comparison of the chromatograms of the Ehrlich-positive peptides derived from hyaluronidase and lysozyme with papain digestion.

Preparative chromatograms were run with butanol-acetic-acid-water (60:15:25) in Whatman No. 1. paper using ascending arrangement. Fig. 2 shows the Ehrlich-positive fractions. With ninhydrin staining several additional fractions could be observed from both chromatograms. Papain itself did not give any colour with Ehrlich's reagent. The positive bands were located, cut and eluted with water.

*Continued Degradation with Chymotrypsin.* — The purified Ehrlich-positive fractions were treated with chymotrypsin (conc. 6.5 mg.%) for 24 hr. + 37°C. at pH 8 obtained by the addition of phosphate buffer. The new digests were lyophilized. New preparative chromatograms (Fig. 3) were run for 36 hr. as above but using descending system. In a control chromatogram, prepared from chymotrypsin, faint Ehrlich-positive spots were obtained, rather similar to those from lysozyme. The new Ehrlich-positive fractions were again eluted with water and the solutions concentrated to 10 ml.

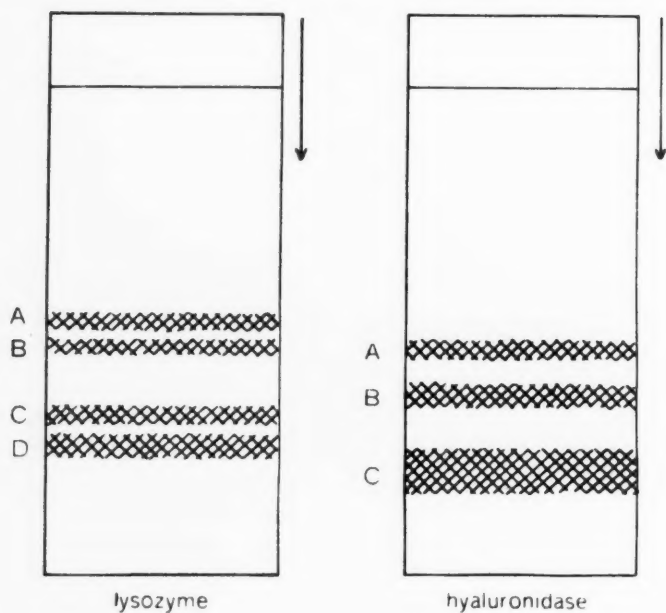


Fig. 3. — Comparison of the chromatograms of the Ehrlich-positive peptides derived by chymotrypsin digestion from the fractions shown in Fig. 2.

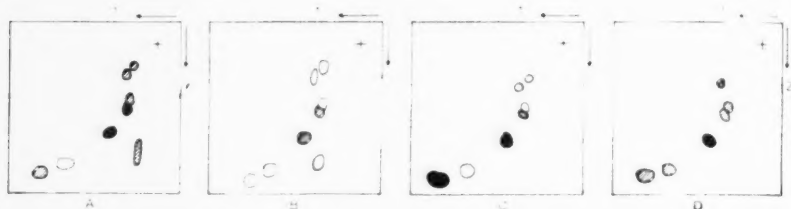


Fig. 4. — Amino acid chromatograms of the peptides from lysozyme, shown in Fig. 3.

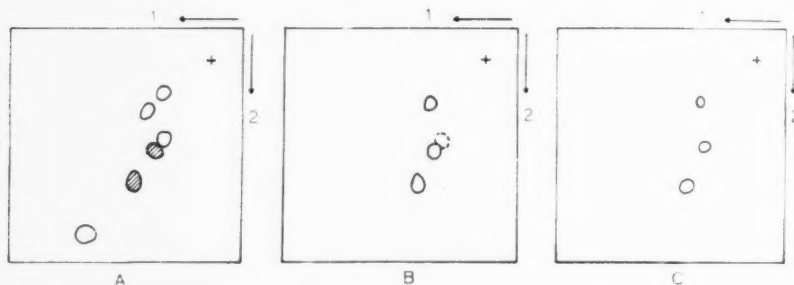


Fig. 5. — Amino acid chromatograms of the peptides from hyaluronidase, shown in Fig. 3. Solvent 1: butanol-acetic acid-water, solvent 2: aqueous phenol-NH<sub>3</sub>.



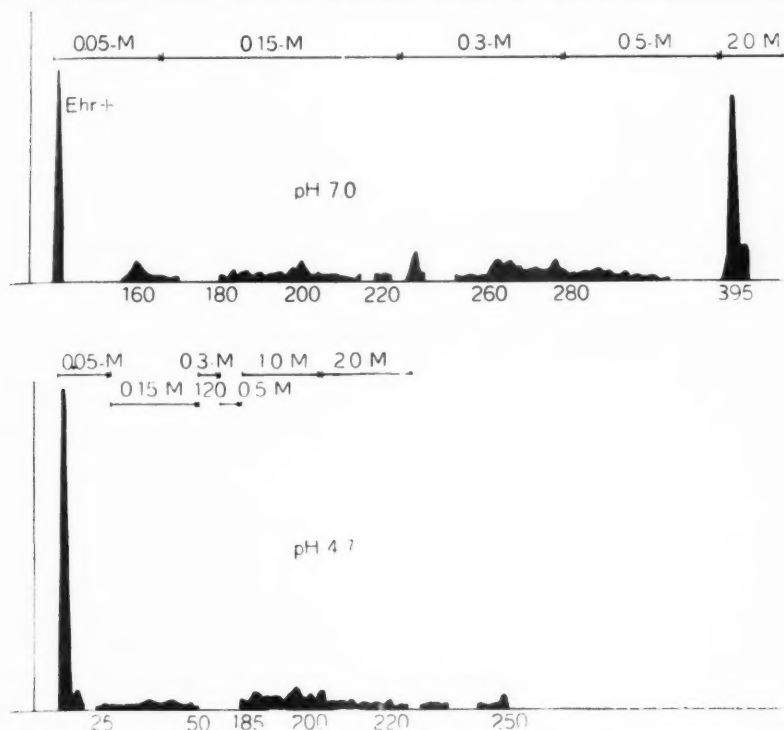


Fig. 6. — Fractionation pattern of the enzymic digests of hyaluronate, made according to Weissmann *et al.* (3). The concentrations refer to the eluent, formic acid. The fraction numbers are indicated.

*Final Hydrolysis of the Ehrlich-positive Fractions of Hyaluronidase and Lysozyme with Barium Hydroxide.* —  $\text{Ba}(\text{OH})_2$  was added to 14% concentration and the solution kept at boiling water bath under reflux and nitrogen atmosphere for 18 hr. These precautions were taken to preserve the tryptophan. The  $\text{Ba}(\text{OH})_2$  was then neutralized with *N* sulphuric acid using methyl red as the indicator. The precipitate was washed with hot water and the washing combined to the supernatant, which was evaporated at a boiling water bath under the atmosphere of nitrogen. The residue was dissolved into 10% isopropanol and filtered. Two-dimensional chromatograms were prepared using ascending system, butanol-acetic acid-water mixture as the first solvent and water-saturated phenol (containing 1% ammonia) as the second. Between and after the runs the papers were dried at  $+80^\circ\text{C}$ . and finally stained with ninhydrin. Figures 4 and 5 show the amino acid composition of the fractions. An Ehrlich-positive spot corresponding to tryptophan was obtained in the lysozyme fractions B and C.

*Preparation of the Enzymic Digest from Hyaluronate.* — Concentration of hyaluronate was 0.2% and of hyaluronidase 0.002%. The hydrolysis was carried out both at pH 4.7 (acetate buffer, 0.2 *M*) and at pH 7.0 (phosphate buffer, 1/15 *M*) and it lasted for 6 days at + 37°C. The chromogen had then reached the maximum in pH 7 sample. The fractionation of the digests according to Weissmann, Meyer, Sampson and Linker (1954) is seen in the Figure 6 and the ordinate shows the colour obtained with carbazol reaction by Dische. The only Ehrlich-positive fraction was the washing fluid of the pH 7 hydrolyzate. At pH 4.7 no chromogen was present and it was not formed in 48 hr. by mere adjustment of pH to 7.

Several of the fractions were hydrolyzed with 6 *N* hydrochloric acid for chromatographic amino acid analysis. All the fractions contained amino acids. The washing fluids contained approximately equimolar amounts of glucosamine and glucuronic acid. They also contained amino acids but the chromatograms were not satisfactory for identification, because of abundant salt.

The elution pattern is different from that presented by Weissman *et al.* (3), which might be due to the long duration of hydrolysis.

#### DISCUSSION

These experiments do not support the idea of similarities in the tryptophan peptides of hyaluronidase and lysozyme. The contribution of tryptophan to the positive colour reaction of *p*-dimethylaminobenzaldehyde with enzymic digests of hyaluronate has not been previously appreciated.

#### SUMMARY

Testicular hyaluronidase preparation, purified with electrophoresis, contained tryptophan, which contributed to the red colour given by the enzymic hydrolyzates of hyaluronate with *p*-dimethylaminobenzaldehyde. The enzyme preparation studied contained also iron. The chromogen present in partial enzymic hydrolyzates of hyaluronate was not attached to a basic resin.

Thryptophan-containing peptides of hyaluronidase and lysozyme were studied and compared.

*Acknowledgement.* — We are indebted to »Sigrid Jusélius Foundation» for institutional support during this work.

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## BONE PATTERN IN EXPERIMENTAL OSTEOPOROSIS OF THE RAT

ROENTGENOLOGIC STUDIES

by

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There is an increasing literature dealing with pathological and experimental changes in the mineral content of human and animal bones. With laboratory animals it is possible to study the entire skeleton, and the results obtained with radiological methods can easily be compared with the true mineral contents of bones.

The best way to produce an experimental osteoporosis is to feed laboratory animals with a diet containing an inadequate amount of calcium and D-vitamin. Gershon-Cohen and McClendon (6, 8) showed that a low calcium diet fed to weanling rats produced radiologically evident osteoporosis in two months, while the same diet fed to adult rats did not cause notable changes even after six months, owing to the large natural stores of bone minerals in adult animals.

The development of osteoporosis in different bones of the skeleton is open to question (3, 4, 5, 10, 12, 15). Therefore, the purpose of this work was to examine the demineralization caused by a low calcium diet in various bones of the rat, and to study the roentgenologic detectability of these changes with the aid of an analysis of the morphological bone pattern.

## MATERIAL AND METHODS

The series consisted of 20 weanling white male rats of the same weight. Ten of these formed the control group receiving ordinary food, rich of vitamins and minerals. The test group was fed with the following mixture:

|                          |           |
|--------------------------|-----------|
| Potato starch .....      | 100 parts |
| Casein .....             | 12 »      |
| Sodium chloride.....     | 1 »       |
| Potassium chloride ..... | 1 »       |

The drinking water was found to contain 0.001 per cent of calcium.

Two rats, one belonging to the test group, the other to the control group were sacrificed every four weeks. Thus, the entire investigation lasted for 40 weeks. The carcasses were radiographed side by side on a fine-grained non-screen roentgen film, Kodak's Crystalex. A Siemens' Fine Structure roentgen tube, AG W 3ö, equipped with a copper anode and beryllium window was used. The focus-film distance was 75 cm, and the tension 40 kV. An aluminium step-wedge was used as a blackening standard. This contained ten steps of the thickness of 1 mm. The films were developed under standardized conditions in Kodak's D76 fine-grain developer for 12 min with continuous agitation. When the blackening of the first film was inadequate or uneven, several films were exposed and developed, and only films showing an uniform blackening were used. These roentgenograms were magnified with the aid of an ordinary photographic enlarger by five diameters. A special exposure meter (Majosix, Gossen) was used. The density measurements of the images to be magnified were made on metaphyses of the long bones and on the middle of the vertebrae, and the exposure was adjusted so as to give the same density of the enlarged images. Kodak's Kodaline Standard film was used for the enlargements.

The following bones were examined: both humeri, tibiae and femurs, three caudal thoracic and lumbar vertebrae and three cranial coccygeal vertebrae (Figs 1, 2, 5 and 6). The magnified images of the bones were analysed visually and by line scanning with the aid of a double-beam recording densitometer (Joyce &



Fig. 1.

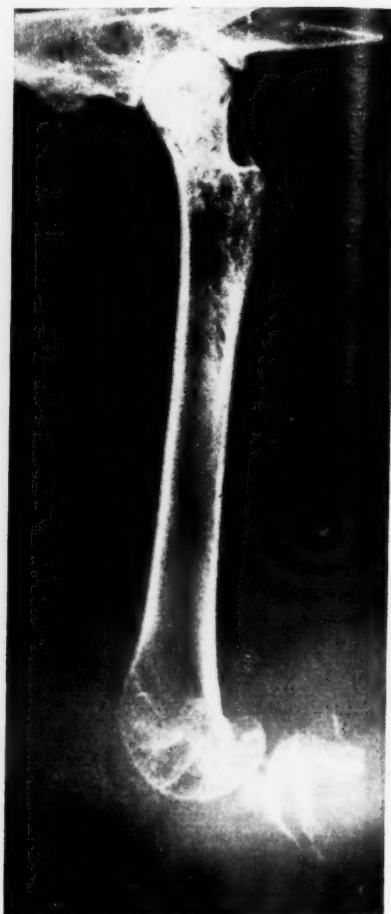


Fig. 2.

Fig. 1. — Femur of a normal rat.

Fig. 2. — Osteoporotic femur of a rat fed with a low calcium diet for 9 months. Decrease in the mineral content was 18.2 per cent.

Loebl and Co., Ltd., Newcastle upon Tyne, England). A round spot of the diameter of 1 mm was used in the scanning. The ratio of the lever connecting the densitometric table and the recording pen was 1 to 20, and thus a recording magnification by 20 diameters was obtained.

Measurements were carried out along tracing paths across the diaphyses of the long bones. Three tracing paths were recorded in

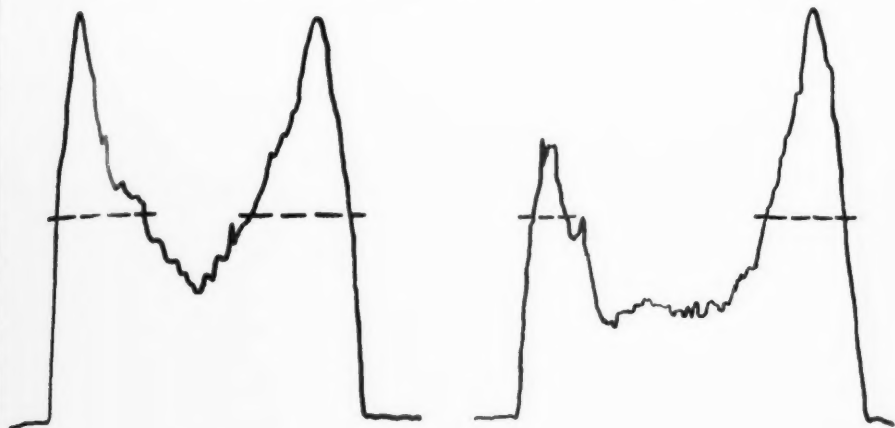


Fig. 3.

Fig. 4.

Fig. 3. — Densitometric curve obtained from the diaphysis of the normal femur seen in Fig. 1. The peaks are caused by the cortical layers. Their widths were measured at a distance of 2.5 cm from the basal line, and the actual thickness of one cortical layer was used in the calculations. The value obtained in this case was 0.6 mm.

Fig. 4. — Densitometric curve obtained from the diaphysis of the osteoporotic femur seen in Fig. 2. The cortical thickness was 0.27 mm.

each case. There were two deflections in each curve caused by the cortical layer. The widths of these deflections were measured (Figs 3, 4), and the thickness of the cortical layer of the bone was obtained by calculating the arithmetic mean of these values. The vertebrae were analysed in the same manner (Figs. 7, 8). The widths of the peaks caused by the trabeculae were measured at a distance of 1 cm from the basal line, and the percentage width of all the peaks of the total length of the scanning line was calculated. The trabecular ratio of a vertebra was obtained by calculating the arithmetic mean of the values obtained from three scanning lines recorded across each vertebra examined.

After radiography, the bones were removed from the carcasses. They were cleaned and degreased with benzene, and their volume was measured pycnometrically using a method described in an earlier paper (7). The bones were then exposed to 1000°C in an electric oven for five hours, and the remaining ash was weighed. The mineral contents of the bones were calculated by dividing the ash weight by the volume.

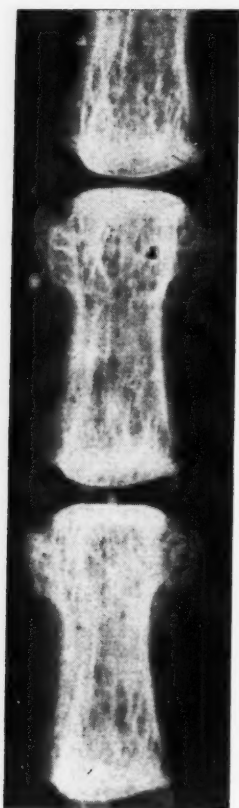


Fig. 5.

Fig. 5. — Normal coccygeal vertebrae of the rat.



Fig. 6.

Fig. 6. — Osteoporotic coccygeal vertebrae of a rat fed with the low calcium diet for 9 months. Decrease in the mineral content was 23 per cent.

#### RESULTS

The results obtained from rats of the test group and those of the control group every four weeks were compared with each other. Variations in the ash contents of the long bones and, on the other hand, of the vertebrae were rather uniform. Therefore their arithmetic means were used in further calculations (Table 1). There were no marked difference in the ash contents of the bones between the test group and the control group after 4 months. After six months, the decrease in the ash content was 7.8 per cent in the





Fig. 7. — Densitometric curve obtained from the normal vertebrae seen in Fig. 5. — The trabecular ratio was 69 per cent. It was obtained by calculating the percentage ratio of the total width of the peaks caused by the trabeculae to the total length of the line drawn at a distance of 1 cm from the basal line.



Fig. 8. — Densitometric curve obtained from the osteoporotic vertebrae seen in Fig. 6. The trabecular ratio was 26.5 per cent.

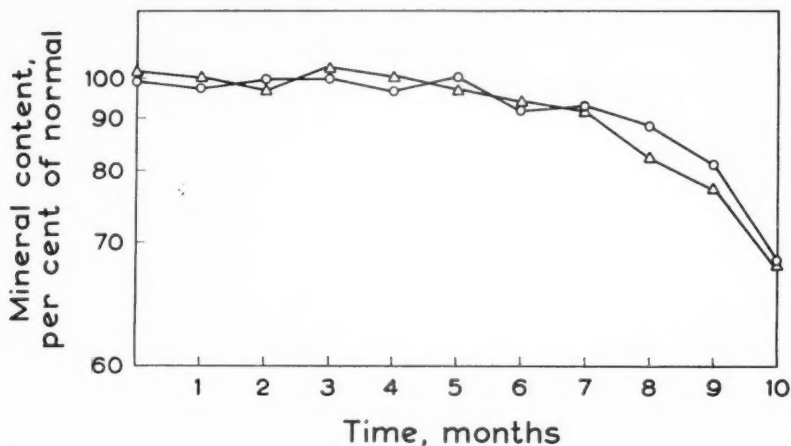


Fig. 9. — Decrease of the average mineral contents of the bones examined during the low calcium diet. Long bones: curves with open dots. Vertebrae: Curves with triangles.

TABLE 1  
ASH CONTENTS OF THE BONES EXAMINED, G CM-3

| Time elapsed, months | 2         | 3         | 4         | 5         | 6         | 7         | 8         | 9         | 10        |
|----------------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
|                      | a         | a         | a         | a         | a         | a         | a         | a         | a         |
|                      | b         | b         | b         | b         | b         | b         | b         | b         | b         |
| Tibiae .....         | .382 .384 | .380 .382 | .378 .383 | .380 .383 | .372 .382 | .372 .386 | .354 .390 | .302 .384 | .301 .390 |
| Femurs .....         | .388 .384 | .382 .379 | .379 .391 | .372 .384 | .361 .390 | .368 .389 | .348 .386 | .316 .386 | .264 .378 |
| Humeri .....         | .386 .372 | .377 .382 | .372 .383 | .370 .390 | .364 .382 | .348 .382 | .334 .387 | .332 .387 | .284 .385 |
| Mean of long bones   | .385 .378 | .379 .381 | .377 .386 | .376 .386 | .366 .386 | .362 .386 | .346 .388 | .316 .386 | .263 .385 |
| Vertebrae:           |           |           |           |           |           |           |           |           |           |
| Thoracic .....       | .371 .370 | .382 .376 | .381 .371 | .361 .381 | .352 .391 | .362 .383 | .308 .376 | .293 .379 | .262 .375 |
| Lumbar .....         | .372 .373 | .372 .364 | .373 .382 | .364 .378 | .343 .386 | .352 .376 | .312 .384 | .290 .381 | .257 .378 |
| Coccygeal .....      | .378 .376 | .371 .376 | .372 .371 | .368 .374 | .356 .378 | .362 .378 | .314 .389 | .291 .376 | .249 .370 |
| Mean of vertebrae    | .374 .373 | .375 .372 | .376 .375 | .365 .378 | .351 .386 | .359 .379 | .312 .382 | .292 .379 | .256 .378 |

a = test group, b = control group.

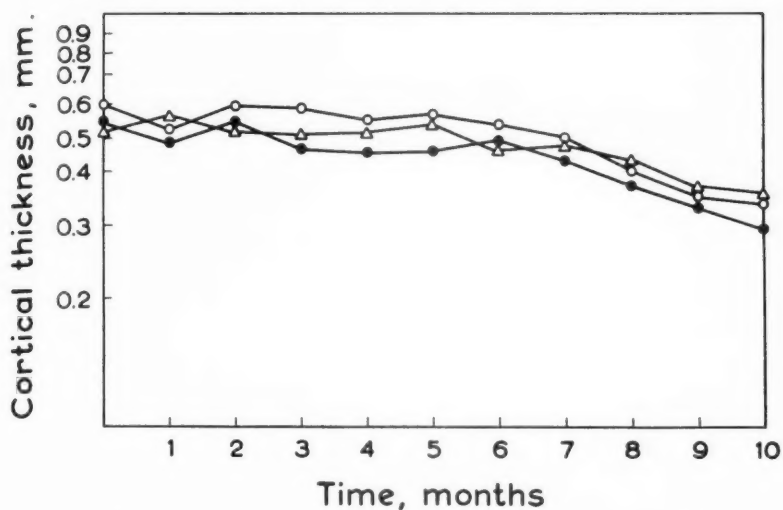


Fig. 10. — Decrease of the cortical thickness of the long bones caused by the low calcium diet. Tibiae: Curves with open dots, Femurs: Curve with triangles. Humeri: Curve with closed dots.

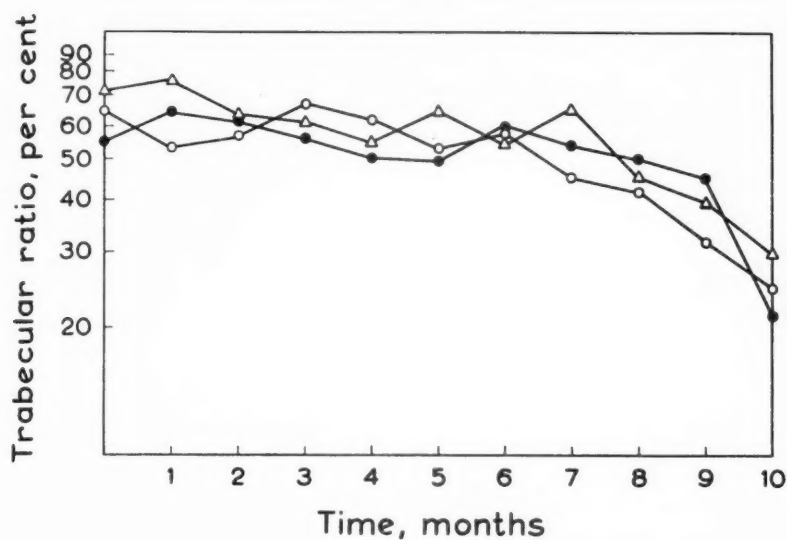


Fig. 11. — Decrease of the trabecular ratio of the vertebrae caused by the low calcium diet. Thoracic vertebrae: Curve with open dots. Lumbar vertebrae: Curve with closed dots. Coccygeal vertebrae: Curve with triangles.

long bones and 9 per cent in the vertebrae. After 8 months, the rate of demineralization increased. (Fig. 9). The cortical thickness of the long bones started to decrease after 7—8 months, after a demineralization of about 10—15 per cent. (Fig. 10). Variations in the trabecular ratio of the vertebrae examined was more marked than those in the cortical thickness of the long bones, and after 7 months there was a decrease in the average values calculated (Fig. 11).

Visual assessment of the degree of demineralization proved to be very unreliable, and changes less than about 20 per cent could not be detected.

#### DISCUSSION

Osteoporotic changes in the skeletons of ten weanling rats fed with a low calcium diet started slowly, and the natural stores of bone minerals were apparently not used until after seven months. After this time the rate of demineralization increased, and after nine months, when the loss of bone minerals was about 20 per cent, osteoporosis was even visually discernible. This is in accordance with the results obtained by theoretical calculations (13). The mineral concentration of the vertebrae was slightly smaller than that of the long bones, but the loss of bone minerals was of the same order in both. No special site of predilection of osteoporosis could be found in the course of this study. The rate of demineralization was similar in all the bones examined. This is in accordance with the results obtained by Ellinger and others (5), but in controversy with the results obtained by analysing the mineral contents of various human bones (3, 10, 12, 15). However, functional demands may be essentially different in laboratory animals and in man.

Thickness of the cortical layer of the long bones is relatively easy to measure, and it can be used as an estimate of the mineralization stage even in clinical radiology (1, 16). However, it is not a sensitive indicator of demineralization, and a reduction in the cortical thickness is not apparent until the loss of minerals is more than 10 to 15 per cent.

Length and volume of the bones were not appreciably altered in the rats fed with the low calcium diet, as earlier shown by Bell and all. (2).

The trabecular ratio was a relatively unreliable estimate of

of the mineralization grade. It showed a reduction at about the same stage as the cortical thickness, but there was a marked variation in the values obtained. The total magnification by 100 diameters, obtained by a photographic enlargement by 5 diameters and recording enlargement by 20 diameters, seems necessary because of superimposing of the trabeculae on each other. Therefore, the bones examined by this method must be relatively thin (14, 17).

Analysis of the morphological pattern of bone is relatively simple as compared with the densitometric measurement of variations of the photographic density caused by absorption changes of the bone tissue. Moreover, there are no errors caused by the uneven distribution of the bone minerals or by the covering effect of the non-mineralized tissue, when the morphological changes of bones are analysed.

On the other hand, results obtained by measuring changes in the roentgen absorption of the bone are more sensitive indicators of demineralization (9, 11). Both of these methods are superior to the visual assessment of osteoporosis. Therefore, depending on the technical facilities available, measurements of the film blackening or analysis of the morphological pattern of the bone tissue should be used instead of the visual assessment even in clinical radiology of osteoporosis.

#### SUMMARY

A low calcium diet was fed to 10 weanling rats, and development of osteoporosis was examined by comparing the ash contents and radiological appearances of the bones of these rats with those of normal rats every four weeks. The loss of bone minerals was almost 10 per cent after six months, the rate of demineralization increasing rapidly after seven months. Decrease in the cortical thickness of the long bones and in the relative amount of trabeculae in the vertebrae was detected by line scanning with a recording densitometer from enlarged roentgenograms after a demineralization of about 10–15 per cent, the changes being not visually discernible until after a mineral loss of about 20 per cent.

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## SEROLOGIC TESTS IN FRESH CASES OF PULMONARY TUBERCULOSIS TREATED BY CHEMOTHERAPY

by

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Serologic tests for tuberculosis have not — at least so far — attained importance in clinical work (1—4). However, they are interesting in theory and the mechanisms on which they are based are not well known. Examinations using different tests may give variable results for the same patient, and therefore it may be postulated that parallel tests carried out by different techniques during the course of the disease process may throw light on the principles underlying the tests.

In the present work, three different serologic tests were performed monthly in fresh cases of pulmonary tuberculosis during 8 months after their hospitalization. During that time all the patients examined received combined antituberculous chemotherapy. The series consisted of 61 adult patients with pulmonary tuberculosis, which was either a recently diagnosed and still untreated process, or a recently diagnosed relapse following an earlier, apparently arrested process. Roentgenologically the tuberculous process was evaluated as far advanced in 33 cases and as moderately advanced in 28 cases. Tubercle bacilli had been found in the sputum of all patients at the time of commencement of the chemotherapy or shortly preceding it.

During the hospitalization the following examinations were made monthly: ESR, Tb staining from sputum by Ziehl-Neelsen

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<sup>1</sup> Aided by a grant from the Sigrid Jusélius Foundation.

method, determination of C-reactive protein by the APC test (8), Middlebrook-Dubos hemagglutination test, Meinicke test for tuberculosis and a serologic test for tuberculosis developed in the State Serum Institute and here called the VSL test (7). ESR was regarded as elevated if it was 15 mm/1 hr. or more. A positive Middlebrook-Dubos test was one in which the titer was 1/16 or higher.

It was our intention to have all the patients under hospital treatment for at least 8 months. However, contrary to our recommendation, some patients left the sanatorium already during the first months. As a result the series decreased continuously in size during the observation period. The patients were therefore grouped according to months of treatment into 9 groups. Thus group 1 included only 2 patients who left during the first month and for whom only the first test results are available, group 2 consisted of 4 patients who wished to be discharged during the second month, and so on, as seen in table 1.

TABLE 1  
DISTRIBUTION OF MATERIAL ACCORDING TO OBSERVATION TIME

| Group No. | Duration of Observation | Number of Cases |
|-----------|-------------------------|-----------------|
| I         | 0—1 months              | 2               |
| II        | 1—2 "                   | 4               |
| III       | 2—3 "                   | 7               |
| IV        | 3—4 "                   | 6               |
| V         | 4—5 "                   | 7               |
| VI        | 5—6 "                   | 7               |
| VII       | 6—7 "                   | 7               |
| VIII      | 7—8 "                   | 10              |
| IX        | 8—9 "                   | 11              |
| I—IX      | 0—9 "                   | 61              |

#### RESULTS

Fig. 1 shows the changes occurring during chemotherapy in ESR, CRP and Tb positivity, expressed as the mean positive value for all patients examined at the time stated. The reliability of the curves decreases towards the end of the observation period because of the decreasing size of the groups. The graph also shows the mean ESR and CRP values for all the patients examined at given times after commencement of the treatment.



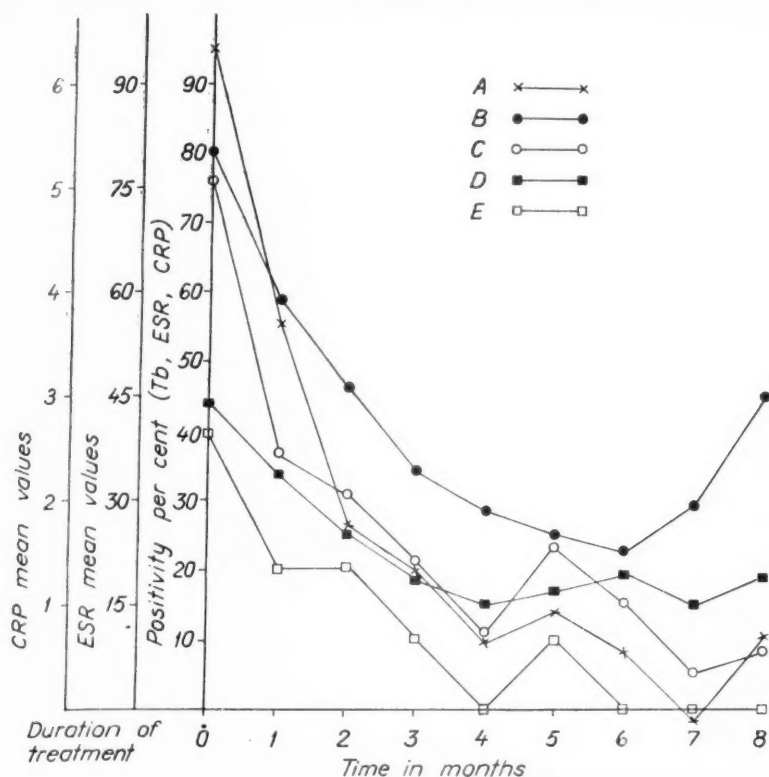


Fig. 1. — Tb (A), ESR (B) and CRP (C) positivity per cent during treatment ESR mean values (D) and CRP mean values (E).

The changes occurring during chemotherapy in the percentages of positivity of Meinicke, Middlebrook-Dubos and VSL tests are given in fig. 2. Fig. 3 shows the means of Middlebrook-Dubos and VSL values, obtained by adding up the titer denominators and dividing the sum by the number of patients examined. Tables 2—4 correspond to these graphs and give the figures on which the curves are based and the number of patients in each group. As is seen from the tables, the serologic tests were performed on only a part of the patients when treatment was commenced. As stated above the latter portion of the curves is likewise not fully reliable in view of the decreasing number of patients with increasing length of observation time.

In the series studied the percentage of Meinicke positive cases during the first 2—3 months of treatment was very high but then

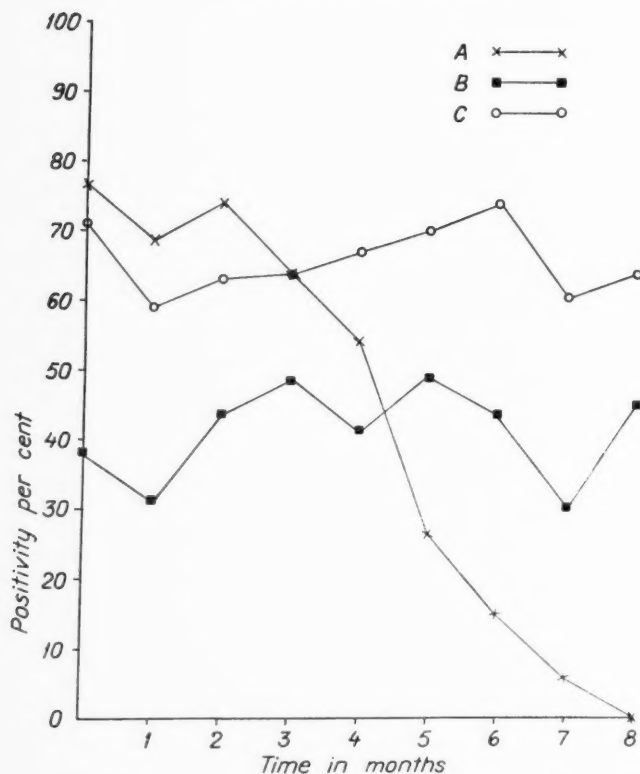


Fig. 2. — Positivity per cent of Meinicke (A), Middlebrook-Dubos (B) and VSL (C) tests during treatment.

TABLE 2

MEINICKE POSITIVITY PER CENT OF ALL CASES EXAMINED

| Group<br>No.             | Duration of Treatment in Months |     |     |    |    |    |    |    |    |  |
|--------------------------|---------------------------------|-----|-----|----|----|----|----|----|----|--|
|                          | 0                               | 1   | 2   | 3  | 4  | 5  | 6  | 7  | 8  |  |
| I                        | 0                               |     |     |    |    |    |    |    |    |  |
| II                       | 100                             | 50  |     |    |    |    |    |    |    |  |
| III                      | 100                             | 57  | 72  |    |    |    |    |    |    |  |
| IV                       | —                               | 20  | 0   | 33 |    |    |    |    |    |  |
| V                        | —                               | 83  | 86  | 43 | 29 |    |    |    |    |  |
| VI                       | 100                             | 100 | 84  | 57 | 43 | 14 |    |    |    |  |
| VII                      | 100                             | 84  | 100 | 86 | 72 | 43 | 17 |    |    |  |
| VIII                     | 100                             | 100 | 84  | 70 | 50 | 22 | 30 | 0  |    |  |
| IX                       | 67                              | 44  | 66  | 72 | 63 | 27 | 0  | 10 | 0  |  |
| I—IX                     | 77                              | 68  | 74  | 63 | 53 | 26 | 15 | 6  | 0  |  |
| No. of cases<br>examined | 13                              | 52  | 42  | 48 | 42 | 34 | 26 | 18 | 10 |  |
| Total                    | 61                              | 59  | 55  | 48 | 42 | 35 | 28 | 21 | 11 |  |

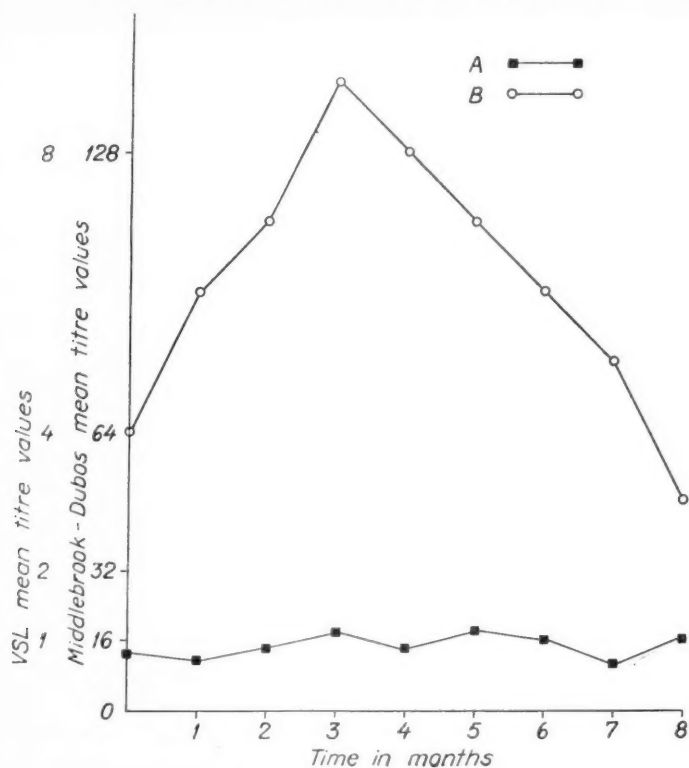


Fig. 3. — Middlebrook-Dubos (A) and VSL (B) mean titre values during treatment.

TABLE 3

MIDDLEBROOK-DUBOS POSITIVITY PER CENT OF ALL CASES EXAMINED

| Group<br>No.             | Duration of Treatment in Months |    |    |    |    |    |    |    |    |  |
|--------------------------|---------------------------------|----|----|----|----|----|----|----|----|--|
|                          | 0                               | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  |  |
| I                        | 50                              |    |    |    |    |    |    |    |    |  |
| II                       | 0                               | 50 |    |    |    |    |    |    |    |  |
| III                      | 100                             | 29 | 29 |    |    |    |    |    |    |  |
| IV                       | —                               | 20 | 33 | 33 |    |    |    |    |    |  |
| V                        | —                               | 33 | 29 | 14 | 0  |    |    |    |    |  |
| VI                       | 50                              | 33 | 43 | 72 | 72 | 43 |    |    |    |  |
| VII                      | 0                               | 33 | 43 | 43 | 29 | 57 | 50 |    |    |  |
| VIII                     | 50                              | 20 | 44 | 40 | 40 | 50 | 40 | 10 |    |  |
| IX                       | 33                              | 40 | 64 | 72 | 54 | 45 | 45 | 50 | 45 |  |
| I—IX                     | 38                              | 32 | 44 | 48 | 41 | 49 | 44 | 30 | 45 |  |
| No. of cases<br>examined | 13                              | 54 | 54 | 48 | 42 | 35 | 27 | 20 | 11 |  |
| Total                    | 61                              | 59 | 55 | 48 | 42 | 35 | 28 | 21 | 11 |  |

TABLE 4  
VSL POSITIVITY PER CENT OF ALL CASES EXAMINED

| Group No.             | Duration of Treatment in Months |    |    |    |    |    |    |    |    |
|-----------------------|---------------------------------|----|----|----|----|----|----|----|----|
|                       | 0                               | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  |
| I                     | 50                              |    |    |    |    |    |    |    |    |
| II                    | 100                             | 75 |    |    |    |    |    |    |    |
| III                   | 0                               | 72 | 86 |    |    |    |    |    |    |
| IV                    | —                               | 0  | 20 | 20 |    |    |    |    |    |
| V                     | —                               | 83 | 83 | 67 | 50 |    |    |    |    |
| VI                    | 100                             | 50 | 72 | 43 | 57 | 72 |    |    |    |
| VII                   | 67                              | 67 | 67 | 84 | 57 | 57 | 50 |    |    |
| VIII                  | 100                             | 50 | 55 | 80 | 80 | 70 | 70 | 50 |    |
| IX                    | 67                              | 60 | 54 | 72 | 81 | 72 | 90 | 70 | 63 |
| I—IX                  | 71                              | 59 | 63 | 63 | 67 | 70 | 74 | 60 | 63 |
| No. of cases examined | 14                              | 54 | 53 | 48 | 42 | 35 | 27 | 20 | 11 |
| Total                 | 61                              | 59 | 55 | 48 | 42 | 35 | 28 | 21 | 11 |

TABLE 5  
MIDDLEBROOK-DUBOS MEAN VALUES FOR ALL CASES EXAMINED

| Group No.             | Duration of Treatment in Months |    |    |    |    |    |    |    |    |
|-----------------------|---------------------------------|----|----|----|----|----|----|----|----|
|                       | 0                               | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  |
| I                     | 9                               |    |    |    |    |    |    |    |    |
| II                    | 2                               | 17 |    |    |    |    |    |    |    |
| III                   | 16                              | 10 | 8  |    |    |    |    |    |    |
| IV                    | —                               | 8  | 16 | 16 |    |    |    |    |    |
| V                     | —                               | 9  | 9  | 7  | 6  |    |    |    |    |
| VI                    | 12                              | 9  | 16 | 14 | 17 | 21 |    |    |    |
| VII                   | 5                               | 11 | 18 | 21 | 15 | 21 | 16 |    |    |
| VIII                  | 9                               | 11 | 10 | 15 | 9  | 9  | 10 | 5  |    |
| IX                    | 27                              | 22 | 21 | 25 | 21 | 20 | 22 | 17 | 16 |
| I—IX                  | 13                              | 12 | 14 | 17 | 14 | 17 | 16 | 11 | 16 |
| N.o of cases examined | 13                              | 54 | 54 | 48 | 42 | 35 | 27 | 20 | 11 |
| Total                 | 61                              | 59 | 55 | 48 | 42 | 35 | 28 | 21 | 11 |

declined rapidly. The curve thus followed to a great extent the curves showing ESR, Tb and CRP positivity. The curves showing Middlebrook-Dubos and VSL positivity percentages, on the other hand, were nearly constant throughout the observation time. There possible was a slight increase in positivity during the first three months of treatment, followed by a slight drop. The same is also

TABLE 6  
VSL MEAN VALUES FOR ALL CASES EXAMINED

| Group<br>No.            | Duration of Treatment in Months |    |    |    |    |    |    |    |    |  |
|-------------------------|---------------------------------|----|----|----|----|----|----|----|----|--|
|                         | 0                               | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  |  |
| I                       | 1                               |    |    |    |    |    |    |    |    |  |
| II                      | 4                               | 9  |    |    |    |    |    |    |    |  |
| III                     | 0                               | 3  | 4  |    |    |    |    |    |    |  |
| IV                      | —                               | 0  | 1  | 1  |    |    |    |    |    |  |
| V                       | —                               | 11 | 4  | 7  | 5  |    |    |    |    |  |
| VI                      | 12                              | 6  | 10 | 8  | 9  | 9  |    |    |    |  |
| VII                     | 5                               | 12 | 15 | 18 | 10 | 4  | 5  |    |    |  |
| VIII                    | 2                               | 7  | 7  | 9  | 8  | 7  | 6  | 8  |    |  |
| IX                      | 1                               | 3  | 8  | 12 | 8  | 7  | 6  | 2  | 3  |  |
| I—IX                    | 4                               | 6  | 7  | 9  | 8  | 7  | 6  | 5  | 3  |  |
| No of cases<br>examined | 14                              | 54 | 53 | 48 | 42 | 35 | 27 | 20 | 11 |  |
| Total                   | 61                              | 59 | 55 | 48 | 42 | 35 | 28 | 21 | 11 |  |

true of the mean titers. The individual values for a very large proportion of patients in the Middlebrook-Dubos and VSL tests remained negative throughout the observation period. In a part of cases the Middlebrook-Dubos values were at a constant high level throughout, whereas in other cases there was a fairly definite increase during 2—3 months, followed by a slight decline; this shape of the curve was even more common and more pronounced in the VSL test. The VSL test gave more positive results than the Middlebrook-dubos test. In another study we have obtained the same result and in addition observed that healthy persons give fewer false positive results in the VSL test (5).

Some workers have found a correlation between ESR and the Middlebrook-Dubos hemagglutination test in tuberculous series (3,6). The results obtained in the present study indicate that this may be the case early in the disease under treatment but not later. Meinicke positivity, on the other hand, occurs at the same time as the positivity of Tb bacilli findings and the positivity of ESR and CRP. If the lung process does not react favorably to chemotherapy, ESR also remains high in many cases, and in these more chronic cases the ESR and the Middlebrook-Dubos test may show similarities in respect to the positivity percentage.

## SUMMARY

The Meinicke test, the Middlebrook-Dubos hemagglutination test and a new serologic test for tuberculosis, the VSL test, were carried out during the first eight months of antituberculous chemotherapy in 61 cases of pulmonary tuberculosis. The incidence of positive Meinicke tests was highest during the first 1—3 months of treatment and then declined rapidly, whereas the incidence of positive Middlebrook-Dubos and VSL tests remained more constant and showed a slight tendency to increasing titers during the first few months of treatment.

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## FACTORS AFFECTING THE HUMAN SEX RATIO<sup>1</sup>

by

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In a previous paper (10) we reported a series of 31,215 families with at least two children and analyzed factors affecting the sex ratio. In this series a tendency of repetition of the sex in a family was to be seen. However, the data were considered too limited for discussing the details and the possible existence of some hereditary or incompatibility factors.

Since the publication of the previous paper we have collected further data. In this paper the complete cumulated series, presented in Table 1, as well as some data by Geissler (5), will be analyzed.

### MATERIAL

Our figures are collected mainly from parish registers and partly from current biographies and maternity station records. Families with two, three, four, or five children are recorded without excluding any of the children. Families with more than five children are also included, but not their sixth, seventh, *etc.* children. Families with twins, with known illegitimate children or with children from the mother's previous marriage have been excluded, as have families with stillbirths of unknown sex.

<sup>1</sup> Supported in part by the Sigrid Jusélius Foundation.

TABLE 1  
SEQUENCES OF SEXES AMONGST THE FIRST N CHILDREN IN FAMILIES OF N OR MORE CHILDREN

| N = 2        | N = 3        | N = 4        | N = 5        | N = 5 (cont.) |
|--------------|--------------|--------------|--------------|---------------|
| Sequence No. | Sequence No. | Sequence No. | Sequence No. | Sequence No.  |
| mm 13552     | mmm 4642     | mmmm 1534    | mmmmm 549    | fmmmm 186     |
| mf 12597     | mmf 4510     | mmmf 1457    | mmmmf 516    | fmmmf 147     |
| fm 12882     | mfm 4175     | mmfm 1459    | mmmfm 478    | fmmfm 179     |
| ff 12837     | mff 4152     | mfff 1433    | mmfff 495    | fmmff 152     |
| Total 51868  | fmm 4240     | mfmfm 1311   | mmfmm 524    | fmmfm 492     |
|              | fmf 4009     | mfmf 1357    | mmfmf 469    | fmfmf 130     |
|              | ffm 4276     | mffm 1389    | mmffm 476    | fmffm 169     |
|              | fff 4343     | mfff 1360    | mmfff 509    | fmfff 429     |
|              | Total 34347  | fmmmm 1352   | mfmmmm 472   | ffmmmm 502    |
|              |              | fmmmf 1337   | mfmmf 441    | ffmmf 140     |
|              |              | fmfm 1328    | mfmf 456     | ffmf 445      |
|              |              | fmff 1240    | mfmff 453    | ffmff 127     |
|              |              | ffmm 1366    | mffmm 496    | fffmm 155     |
|              |              | ffmf 1300    | mffmf 473    | ffmf 461      |
|              |              | fffm 1358    | mfffm 495    | ffffm 188     |
|              |              | ffff 1487    | mffff 440    | ffff 518      |
|              |              | Total 22068  |              | Total 15162   |

As shown by Table 2 there are significant differences in the sex ratio (number of male births per 100 female births), between different subgroups of the series and between births of different parity. The latter discrepancy may be explained partly by the former; the part of the series obtained from the parish registers

TABLE  
THE SEX RATIOS AT DIFFERENT SUBDIVISIONS OF THE

| Subdivision                           | First Child |           | Second Child |           | Third Child |           |
|---------------------------------------|-------------|-----------|--------------|-----------|-------------|-----------|
|                                       | Number      | Sex Ratio | Number       | Sex Ratio | Number      | Sex Ratio |
| Material of the previous paper (8) .. | 31215       | 101.2     | 31215        | 101.8     | 18589       | 100.4     |
| Maternity stations ..                 | 3150        | 98.5      | 3150         | 112.3     | 2168        | 100.2     |
| Biographies .....                     | 9154        | 101.9     | 9154         | 105.8     | 7483        | 105.5     |
| Parish registers ....                 | 8349        | 104.5     | 8349         | 107.0     | 6107        | 102.8     |
| Total                                 | 51868       | 101.7     | 51868        | 103.9     | 34347       | 101.9     |



and biographies has a high sex ratio and a strong representation in the large families.

Our heterogeneous series would not be suitable for comparisons between the actual distribution of different types of families (say, male-female-male-female) and the distribution which one would expect supposing that the (constant) sex ratio and chance are the only determining factors. For this reason, and for the reason given below we have not carried out this type of «binomial analysis», but have only studied the plain sex ratios. Thus we believe that the heterogeneity of our series is not fatal to the conclusions, even though the pooled series was used in the analysis.

An additional reason for us to reject the «binomial analysis» was that family planning, so far as it is practiced, affects the random distribution in an unknown way. That family planning affects our figures can be seen from Table 3, which gives some «fertility figures» (the proportion of families with children who have acquired a further child).

TABLE 3

EFFECT OF THE SEX COMPOSITION OF A FAMILY ON ITS FURTHER FERTILITY

| Type of Family             | Number of Families | Percentage of Families Having a Further Child |
|----------------------------|--------------------|---|
| Unisexual (mm or ff) ..... | 26,389             | 67.5  |
| Bisexual (mf or fm) .....  | 25,479             | 65.0  |

2

MATERIAL AND AT BIRTHS OF DIFFERENT PARITY

| Fourth Child |              | Fifth Child |              | Total  |              | Fertility<br>(Percentage of<br>Families Having at<br>last Five Children) |
|--------------|--------------|-------------|--------------|--------|--------------|--|
| Number       | Sex<br>Ratio | Number      | Sex<br>Ratio | Number | Sex<br>Ratio |  |
| 10530        | 101.5        | 6116        | 100.7        | 97665  | 101.2        | 19.6   |
| 1217         | 94.1         | 1121        | 105.3        | 10806  | 102.9        | 35.6   |
| 5922         | 99.5         | 4884        | 107.7        | 36597  | 104.0        | 53.4   |
| 4399         | 104.8        | 3041        | 109.1        | 30245  | 105.3        | 36.4   |
| 22068        | 101.1        | 15162       | 104.9        | 175313 | 102.6        | 29.2   |

## ANALYSIS OF THE DATA

The probability of a coming child being a boy (hence called  $p$ )<sup>1</sup> must be affected by an interaction of numerous factors in the mother, father and fetus, but we will limit our study to factors which lend themselves to an analysis. We will consider evidence in our data and in other authors' data which suggests the existence of two kinds of factors affecting  $p$ :

1) Factors which are inherent in a certain couple and which, more or less constantly, make the  $p$  in this family different from the  $p$  of other families of the same age, child composition, etc. If families exist which can only have children of one sex, they would exemplify these factors. These factors would be likely to have some inherited background either in the father or in the mother or in both and will be called *inherited factors* in this paper.

2) Factors which the couple has acquired during the reproductive period. These *acquired factors* would be expected to manifest themselves better in the late than in the early part of the reproductive period. We will try to determine whether the sex of elder siblings is such a factor.

*Inherited Factors.* — Several workers (2, 6, 15) favour the view that  $p$  is different in different families of the same population. This seems to be a reasonable assumption; if different races have different sex ratios even if they live in the same country (12), it seems unlikely that any one of the races would be completely homogeneous in this respect.

The above assumption can explain the discovery in our data that adjacent births in a family tend to be of the same sex. If different families of the same population can have a different  $p$  before they start their reproductive activity, and if individual  $p$  values are distributed on each side of the average sex ratio of the population, then, in a large sample of this population boys will have a slight tendency to be followed by a boy and girls by a girl. As can be seen from Table 4, this is the case in our data.

The second children are exceedingly often of the same sex as the first; the sex ratio of the second child is 107.6, if the first child is a boy, and 100.4 if it is a girl. A corresponding difference can be

<sup>1</sup> The numerical approximation of  $p$  in a certain situation is, of course, dependent on the quantity and quality of information available.

seen in Geissler's (5) data (the ratios are 108.0 and 103.1) and in the data of Edwards and Fraccaro (3) (111.4 and 104.7). The fact that this repetition tendency is less remarkable in families of high parity may indicate that in them counteracting (perhaps acquired) factors are operating.

The repetition tendency seems to be very strong in cases where all the preceding children are of the same sex, but it cannot be seen in families in which *all but one* of the preceding children are of the same sex (Tables 8 and 10). *E.g.*, the fifth child has a great likelihood of being a girl if the four preceding children are all girls, but there is a high sex ratio if three of them are girls and one is a boy. This fact slightly suggests that »gynecophilia» and »androphilia» are not polygenic traits as one might have expected.

We feel that inherited factors affecting the sex ratio exist. Yet, these factors cannot explain all the unexpected characteristics of our data; other factors must exist as well.

There are two further findings which require explanation: one of them is that the repetition tendency of the sex, found to be fairly strong in pairs of adjacent births (Table 4), is significantly lower in pairs of births which are separated by one, two or three births (Tables 5, 6 and 7).

TABLE 4  
THE SEXES OF PAIRS OF ADJACENT BIRTHS

| Sex of the First Child of the Pair | Sex of the Second Child of the Pair |        |
|------------------------------------|-------------------------------------|--------|
|                                    | Male                                | Female |
| Male .....                         | 31,973                              | 30,244 |
| Female .....                       | 30,653                              | 30,575 |

$$\chi^2 = 21.78 \text{ on } 1 \text{ D.F.}$$

TABLE 5  
THE SEXES OF PAIRS OF BIRTHS SEPARATED BY ONE BIRTH

| Sex of the First Child of the Pair | Sex of the Second Child of the Pair |        |
|------------------------------------|-------------------------------------|--------|
|                                    | Male                                | Female |
| Male .....                         | 18,357                              | 17,800 |
| Female .....                       | 17,835                              | 17,585 |

$$\chi^2 = 1.26 \text{ on } 1 \text{ D.F.}$$

TABLE 6

THE SEXES OF PAIRS OF BIRTHS SEPARATED BY TWO BIRTHS

| Sex of the First Child of the Pair | Sex of the Second Child of the Pair |        |
|------------------------------------|-------------------------------------|--------|
|                                    | Male                                | Female |
| Male .....                         | 9,646                               | 9,353  |
| Female .....                       | 9,213                               | 9,017  |

 $r^2 = 0.20$  on 1 D.F.

TABLE 7

THE SEXES OF PAIRS OF BIRTHS SEPARATED BY THREE BIRTHS

| Sex of the First Child of the Pair | Sex of the Second Child of the Pair |        |
|------------------------------------|-------------------------------------|--------|
|                                    | Male                                | Female |
| Male .....                         | 3,946                               | 3,796  |
| Female .....                       | 3,816                               | 3,604  |

 $x^2 = 0.31$  on 1 D.F.

A more specific unexpected discovery still to be explained is that a certain number of male births seems to decrease the subsequent  $p$  significantly (Tables 9, 10, 11).

We will try to explain these findings assuming the presence of acquired factors.

No *acquired factors* are known to have an effect on  $p$  even though suggestions have been made (10, 11, 13). Schützenberger's (11) suggestion will be discussed later in this paper. Szilard (13) found that the children of aged fathers have a lower sex ratio than those of young ones, and suggested that XY spermatogonia are more likely to be «hit» by harmful agents (irradiation) than XX spermatogonia. He could not differentiate between the effects of paternal age, maternal age and parity, and did not draw final conclusions until more extensive data are available. Renkonen's (10) suggestion was influenced by the discovery of blood group incompatibilities between the fetus and the mother. The rather recently discovered male histo-incompatibility antigens in the mouse and rat (4) give further support to this idea.

If human mothers are immunized to the male antigens of their sons and if this immunization can be harmful to subsequent male fetuses, a male birth can decrease the  $p$  of the following births. A manifest immunization would be an exceptional event, as it is

TABLE 8

THE SEX RATIO OF THE 2ND, 3RD, 4TH, AND 5TH CHILD OF A FAMILY COMPARED WITH THE NUMBER OF BOYS PRECEDING THIS CHILD

| Number of Boys<br>Preceding the Child<br>Whose Sex Is under<br>Test | Sex Ratio and Its Standard Deviation |                 |                 |                 |
|---|--------------------------------------|-----------------|-----------------|-----------------|
|   | 2nd Child                            | 3rd Child       | 4th Child       | 5th Child       |
| 0   | 100.4 $\pm$ 1.2                      | 98.5 $\pm$ 2.2  | 91.3 $\pm$ 3.8  | 94.2 $\pm$ 6.3  |
| 1   | 107.6 $\pm$ 1.2                      | 103.1 $\pm$ 1.6 | 104.7 $\pm$ 2.2 | 106.1 $\pm$ 3.3 |
| 2   | —                                    | 102.9 $\pm$ 2.1 | 99.9 $\pm$ 2.2  | 105.2 $\pm$ 2.7 |
| 3   | —                                    | —               | 105.3 $\pm$ 3.7 | 105.8 $\pm$ 3.2 |
| 4   | —                                    | —               | —               | 106.4 $\pm$ 6.0 |

in the case of Rh-immunization. More than one male fetus could well be the minimum requirement for a harmful immunization.

To study this question we calculated the sex ratios of *e.g.* the fifth children in the families that previously had 0, 1, 2, 3 or 4 males. The results are shown in Table 8. Because all the actual figures can easily be extracted from Table 1, only the sex ratios and their standard deviations are given in Table 8.

It will be seen from this table that unisexual families tend to obtain further children of the same sex. For this reason it seems advisable to exclude the extreme values in each column and compare only the values in the encircled area. This leaves out the second and the third child, because there is nothing to compare. In the column of the fourth child the sex ratio is higher if one of the previous children is a boy than if two of them are boys. This is compatible with the immunization hypothesis. However, the difference between the second and third figure in this column is not statistically significant. Such an immunization effect cannot be seen in the group of the fifth children, but these sex ratios are based on small series and are therefore not particularly informative.

If, instead of studying the sex ratios of the fourth and fifth children separately, we pool these data and study the sex ratio of children following different combinations of three children, the sex ratio after two girls and one boy is significantly higher than that after one girl and two boys (the last columns of Table 9).

Table 9 also shows that after one boy and two girls there is an excess of boys regardless of whether the boy is the first, second

TABLE 9  
THE CUMULATED SEX RATIO OF THE 4TH AND 5TH CHILDREN COMPARED WITH  
THE NUMBER OF BOYS PRECEDING THE 4TH CHILD, AND DEPENDENCE OF THIS  
SEX RATIO ON THE BIRTH ORDER OF PRECEDING BOYS AND GIRLS

| Type of Family | Total Number of 4th and 5th Children | Sex Ratio | Sex Ratio of Cumulated Data | Difference and its Standard Error |
|----------------|--------------------------------------|-----------|-----------------------------|-----------------------------------|
| mmm            | 5,029                                | 103.8     | 103.8                       | 0.021 $\pm$ 0.032                 |
| mmf            | 4,870                                | 102.0     | 101.7                       |                                   |
| mfm            | 4,490                                | 96.6      |                             |                                   |
| fmm            | 4,553                                | 103.6     |                             |                                   |
| ffm            | 4,480                                | 106.7     | 106.8                       | - 0.051 $\pm$ 0.022               |
| fmf            | 4,388                                | 109.1     |                             |                                   |
| mff            | 4,653                                | 104.7     |                             |                                   |
| fff            | 4,770                                | 93.3      | 93.3                        |                                   |

or third of the preceding children. This shows that the excess is not due to a switch of some families from a gynecophilic to an androphilic period, an event postulated by Schützenberger (11).

An analysis of Geissler's (5) series (Table 10) gives results similar to ours but even more decisive. A very high sex ratio is again found in families which only had boys, and a low ratio in families which had produced girls only. If we again exclude these groups which may contain inherently unisexual families and

TABLE 10  
(EXTRACTED FROM GEISLER'S (5) DATA)  
THE SEX RATIO OF THE 2ND, 3RD, 4TH, 5TH, AND 6TH CHILD OF A FAMILY  
COMPARED WITH THE NUMBER OF BOYS PRECEDING THIS CHILD

| Number of Boys Preceding the Child Whose Sex Is under Test | Sex Ratio and Its Standard Deviation |                 |                 |                 |                 |
|--|--------------------------------------|-----------------|-----------------|-----------------|-----------------|
|  | 2nd Child                            | 3rd Child       | 4th Child       | 5th Child       | 6th Child       |
| 0  | 103.1 $\pm$ 0.6                      | 104.8 $\pm$ 1.0 | 101.4 $\pm$ 1.5 | 104.3 $\pm$ 2.4 | 101.6 $\pm$ 3.8 |
| 1  | 108.0 $\pm$ 0.6                      | 106.8 $\pm$ 0.7 | 107.3 $\pm$ 0.9 | 111.7 $\pm$ 1.2 | 108.8 $\pm$ 1.7 |
| 2  | —                                    | 108.9 $\pm$ 0.5 | 102.9 $\pm$ 0.8 | 105.3 $\pm$ 0.9 | 110.9 $\pm$ 1.2 |
| 3  | —                                    | —               | 107.4 $\pm$ 1.4 | 101.4 $\pm$ 1.1 | 100.8 $\pm$ 1.2 |
| 4  | —                                    | —               | —               | 107.0 $\pm$ 2.2 | 101.0 $\pm$ 1.6 |
| 5  | —                                    | —               | —               | —               | 112.8 $\pm$ 3.3 |

TABLE 11

(DATA FROM GEISSLER'S (5) REPORT)

THE SEX RATIO OF THE 4TH AND 5TH CHILD OF A FAMILY COMPARED WITH THE  
NUMBER OF BOYS PRECEDING THIS CHILD

| Number of Boys<br>Preceding the Child<br>Whose Sex Is under<br>Test | Fourth Child |            |                       | Fifth Child  |            |                       |
|---|--------------|------------|-----------------------|--------------|------------|-----------------------|
|   | Sex<br>Ratio | Difference | S.D. of<br>Difference | Sex<br>Ratio | Difference | S.D. of<br>Difference |
| 0   | 101.4        |            |                       | 104.3        |            |                       |
|   |              | —5.9       | 1.9                   |              | —7.4       | 2.6                   |
| 1   | 107.3        | 4.4        | 1.4                   | 111.7        | 6.4        | 1.6                   |
| 2   | 102.4        | —4.5       | 1.7                   | 105.3        | 3.9        | 1.5                   |
| 3   | 107.4        |            |                       | 101.4        | —5.6       | 2.5                   |
| 4   | —            |            |                       | 107.0        |            |                       |

concern ourselves with the encircled area, we find that in the column «4th child» the sex ratio after two preceding boys is significantly lower than after one. In the column «5th child» the sex ratio after three preceding boys is lower than the ratio after two, which in turn is lower than the ratio after one. In the column of the sixth children there is one exception to the above rule, the ratio is still high after two preceding boys. After three preceding boys it is low as is expected. This could be explained by assuming that women who are not easily immunized and thus have less trouble with their pregnancies have larger families than women who are easily immunized. If this is true, the first type of women would be enriched in families with six (and seven etc.) children.

In Table 11 Geissler's data about the fourth and fifth children are further analyzed. All the differences in sex ratios between adjacent groups are statistically significant (2 to 4 times their respective standard errors). These differences are illustrated in Fig. 1.

From our data (Table 8) families with three preceding children are included in Fig. 1 as well as families with three or four preceding children from Geissler's data (Table 11). The standard deviations in our families with four preceding children are so great that the figures may not give us a correct picture.

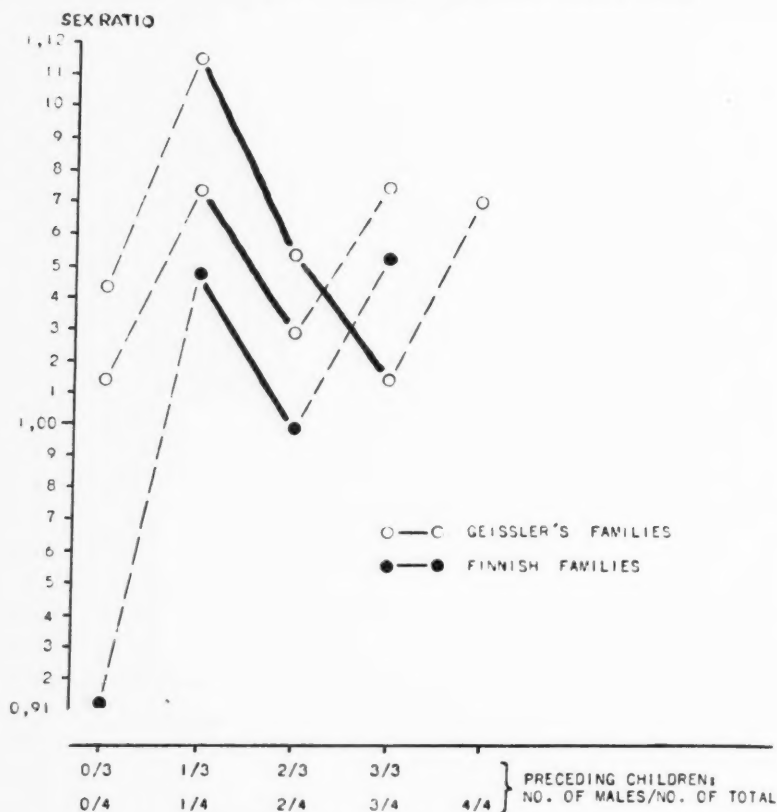


Fig. 1. — The sex ratio of the 4th and 5th child of a family compared with the number of boys preceding this child. For explanations see the text.

The similarity in slope of all three curves is remarkable. This might mean that the immunizing effect of a male pregnancy is similar in the German and Finnish materials. Curves in Fig. 1 seem very difficult to explain by assuming that  $p$  is different in different families and/or that  $p$  varies periodically in a family.

The data can be explained by assuming that among the factors affecting  $p$  in a population there are the following two.

1.)  $p$  is inherently different in different families. The main effect of this factor is a tendency of certain families to obtain only boys and certain others only girls. This tendency is not absolute.

2.) Male pregnancies immunize a small proportion of mothers against male antigens, and this immunization can be harmful to



subsequent male fetuses. The third male fetus of a mother is often affected the fourth still more often, *etc.* Our analysis does not give us a direct answer to the question whether the second and the first boy can also be affected.

The above hypothesis needs the extra assumption that the undisturbed (not disturbed by immunization deaths) sex ratio of, *e.g.*, the fourth and fifth child can be higher than the (undisturbed) sex ratio of the first child. This does not seem inconceivable to us. We are aware that there must be a number of unknown factors affecting the sex ratio. If sex incompatibility is such a factor, other unknown histo-incompatibilities are likely to play a role as well. From blood group incompatibilities we know that even *interfering* interactions occur (ABO- and Rh-incompatibility (7)), which might lead to a very complex effect.

The above hypothesis would lead to certain predictions which lend themselves to study. One of them is that in stillbirths and abortions there should be an excess of boys, and this excess should be greater if boys have been born to the family previously. Some studies on this line have been done but the results are contradictory (1, 14). Another prediction is that the sex ratio should decrease with an increasing parity. There are data indicating that the well-established low sex ratio in old families (8) is dependent on the parental age and birth order rather than maternal age (9).

#### SUMMARY

We have studied factors affecting the human sex ratio by analysing a series of about 50,000 families collected by us, and the series of 108,000 families collected by Geissler (5). We come to the conclusion that some families have a tendency to produce children of one sex. We also suggest that male pregnancies can immunize certain mothers, and this immunization can be harmful to following male fetuses and thus affect the sex ratio.

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## APPENDIX

## SUMMARY OF GEISSLER'S (5) DATA

| Previous children |       | Total Number<br>of Families | Number of Families having, as the<br>following Child, |        |
|-------------------|-------|-----------------------------|---|--------|
| Boys              | Girls |                             | a Boy   | a Girl |
| 1                 | 0     | 114,609                     | 59,518  | 55,091 |
| 0                 | 1     | 108,719                     | 55,196  | 53,523 |
| 2                 | 0     | 47,819                      | 24,923  | 22,896 |
| 1                 | 1     | 89,213                      | 46,078  | 43,135 |
| 0                 | 2     | 42,860                      | 21,928  | 20,932 |
| 3                 | 0     | 20,540                      | 10,634  | 9,906  |
| 2                 | 1     | 57,179                      | 28,997  | 28,182 |
| 1                 | 2     | 53,789                      | 27,843  | 25,946 |
| 0                 | 3     | 17,395                      | 8,759   | 8,636  |
| 4                 | 0     | 8,628                       | 4,459   | 4,169  |
| 3                 | 1     | 31,611                      | 15,916  | 15,695 |
| 2                 | 2     | 44,793                      | 22,970  | 21,823 |
| 1                 | 3     | 28,101                      | 14,825  | 13,276 |
| 0                 | 4     | 7,004                       | 3,575   | 3,429  |
| 5                 | 0     | 3,666                       | 1,943   | 1,723  |
| 4                 | 1     | 16,340                      | 8,210   | 8,130  |
| 3                 | 2     | 30,175                      | 15,144  | 15,031 |
| 2                 | 3     | 28,630                      | 15,053  | 13,577 |
| 1                 | 4     | 13,740                      | 7,159   | 6,581  |
| 0                 | 5     | 2,839                       | 1,431   | 1,408  |

## STUDIES IN THE ELASTICITY OF FROG SKIN <sup>1</sup>

by

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In an earlier study concerning the correlation between the skin stretch of the frog and the electrical output of its sensory skin nerves (1) the elastic properties of the skin constituted a special problem. It was accordingly necessary to study the relationship between the area of the skin and the pressure which was applied on the surface of circular skin strips. Such area-pressure curves of «circular plates» (5) provide information on the elasticity of the material concerned.

The elasticity of the skin is due to the elastic fibres contained in it (6). The elastic tissue gives it a rubberlike nature (9). Like rubber, the skin shows reversible extensibility by virtue of the fibrous proteins, which are principally coiled collagenous fibres with low elastic limits (6). In the same manner as rubber-like substances, the skin also exhibits placticity (for definition of placticity see Goodier and Hodge (3)). Owing to this phenomenon, the skin tissue shows relaxation (decay of stress at constant area) and a creeping effect (increase in area at constant pressure). However, in all structural materials there is an initial part of the stress-strain relationship, in which, for sufficiently small values of the stress, the relationship between stress and strain is linear and reversible. The behaviour of the material is in this instance termed elastic. This paper deals with the elastic behaviour of normal frog skin.

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<sup>1</sup> Aided by a grant from The Finnish State Committee for Natural Sciences.

## METHOD

In order to study the stress-strain relationships in a material such as skin, the most convenient method is that of applying a uniformly distributed pressure on the surface of the skin. This method is commonly used in technical studies of the elasticity of foliated metals, paper etc. (8). In employing this method, an apparatus whose principle is shown in Fig. 1 was used. A cylinder

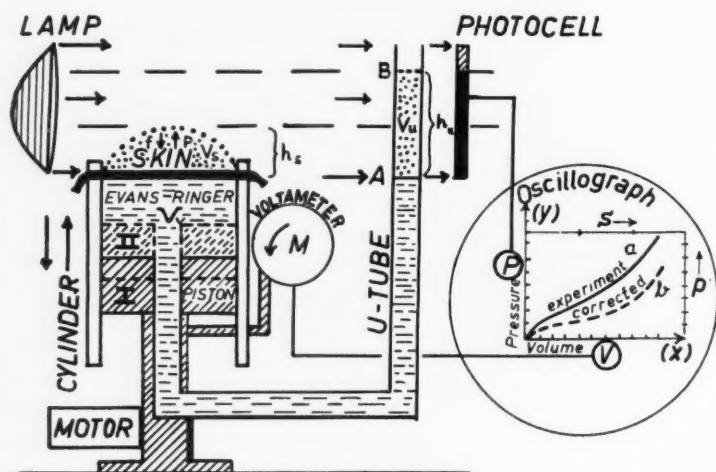


Fig. 1. — Apparatus for recording the stress-strain curves of the frog skin on an oscillograph.

with a moving piston was filled with a solution of frog's Ringer containing Evans Blue. A circular strip of frog's skin was placed on the end of the cylinder and pressed against its border by means of a metal ring in the periphery of the skin. The cavity of the cylinder was connected with an U-tube of glass in which the surface of the Evans-Ringer mixture took the position A, i. e. the same height as the surface of the solution in the cylinder, when the skin was in a position of rest. By moving the piston from position I to position II, the skin was distended as shown by the dotted line in the Figure. According to the elastic force ( $f$ ), with which the skin tended to attain the rest position, the surface in the U-tube increased to the new position B. The situation resulted in an equilibrium between the elastic force ( $f$ ) of the skin, and the pressure ( $p$ ) acting on the surface of the skin against the elastic force. This pressure

was determined by the heights of the Evans-Ringer volumes in the U-tube and under the skin which are situated on the upper side of the resting level A,  $h_u$  and  $h_s$ , shown in the Figure by dotted areas. A lamp was placed on one side of the U-tube, and on the other side a photocell whose voltage output was determined by the height ( $h$ ) of the solution in the U-tube. This voltage was led to the y-axis of a dual beam oscilloscope with 2-dimensional recording-system (type Tektronix 502). The movement of the piston of the cylinder, which is directly proportional to the volume displacement in the system, was led to a potentiometer axis (M) and the resulting voltage (proportional to the movement) to the x-axis of the oscilloscope. The piston was driven by a synchronous motor with adjustable speed.

By moving the piston, the beam of the oscilloscope was deflected in a manner shown in Figure 1 on the right. The x-axis in the oscilloscope represented the volume shift ( $V$ ) in the system and the y-axis the height ( $h_u$ ) of the Evans-Ringer solution in the U-tube. The oscilloscope curves (curve a in Fig. 1) were corrected to real area-pressure curves (curve b in Fig. 1). Fig. 2 shows the

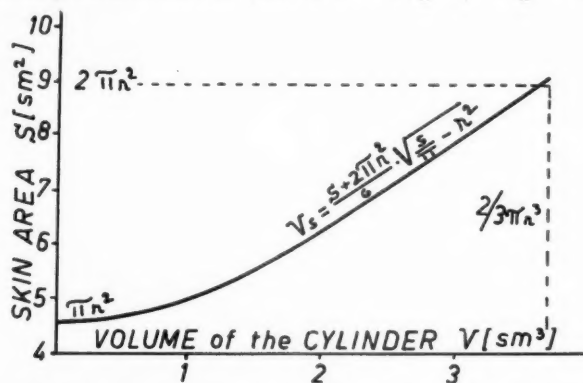


Fig. 2. — The relation between the volume ( $V$ ) of the cylinder (Fig. 1) and the area ( $S$ ) of the skin.

relationship between the volume shift ( $V$ ) and the area ( $S$ ) of the skin. The area was obtained theoretically by assuming that as a result of a slight distension the skin took a spherical shape (the dotted line in Fig. 1) and by using the equation

$$(1) \quad V_s = \frac{S + 2\pi r^2}{6} \cdot \sqrt{\frac{S}{\pi} - r^2}$$

where  $r$  is the radius of the cylinder, and the volume  $V_s = V - V_u$ , where  $V_u$  is the volume shift in the U-tube. The shape taken by the skin under different distensions was checked photographically, and was found to follow equation (1). On the other hand, the pressure ( $p'$ ), shown by the oscillograph, was corrected to the real pressure ( $p$ ) by means of the amount determined by the fluid volume under the skin (height  $h_s$ , measured photographically). As the height of the distended skin ( $h_s$ ) was only 2—4 per cent of height of the fluid in the U-tube, the real pressure was determined as  $(h_u + \frac{1}{2} h_s) = p$  as value which results in an error of less than 3% of  $p$ . The position of the corrected area-pressure curve (b) is shown in relation to the curve (a), which was obtained by the oscilloscope directly from an experiment (in Fig. 1 on the right).

In the experiments to be described here, only area-pressure curves were recorded where the relationship was independent of the time. Thus by moving the piston, its position remained constant until an equilibrium between the forces  $f$  and  $p$  appeared, after which a photograph of the oscillograph screen was taken.

Only fresh preparations of frog skin (*Rana temporaria*) were used. Abnormal skins (diseases etc.) were omitted. Skins taken from both the dorsal and ventral regions of the body were used. The circular skin strips were placed on the cylinder in the rest position, *i.e.* in the same state as they were in situ. This was checked by measuring the position of the pigment configuration before the preparation, and after the insertion on the cylinder. If the skin was left on the cylinder for a prolonged time, frog's Ringer solution was used on the upper surface of the skin. Area-pressure curves were taken up to two days after the skin had been prepared.

The series consisted of more than 30 skin strips of which 15 were from the ventral body surface. More than 20 examinations were made with each preparation. As it was found that the time interval between experiments influenced the results, a time of minimum half an hour was left between two complete recordings of the area-pressure curve. The temperature during these experiments varied from 21° to 24°C.

#### RESULTS

Fig. 3 shows original oscillograph recordings of area-pressure relations of different skin preparations. The horizontal axis

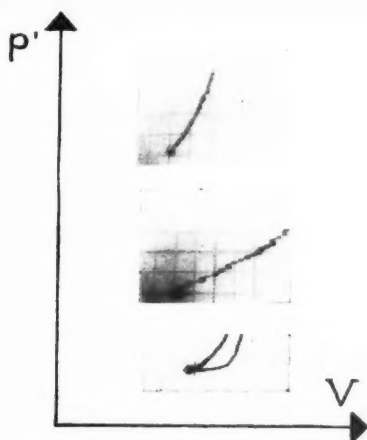


Fig. 3. — Original oscillograph recordings of area-pressure relations of frog skin preparations. Horizontal: volume; vertical: pressure.

represents the volume  $V$ , whose relation to the skin area is given by Figure 2. The vertical axis represents the pressure  $p'$  recorded by the apparatus used.

Fig. 4 shows one corrected area-pressure curve. On the horizontal axis is the distended skin area per unit undistended area ( $S/S_0$ ) and on the vertical axis the pressure  $p$  acting on the skin surface.

Fig. 5 represents four area-pressure curves of the same preparation (dorsal skin) taken within 24 hours, the time interval between each of the curves being 8 hours. The representation is three-

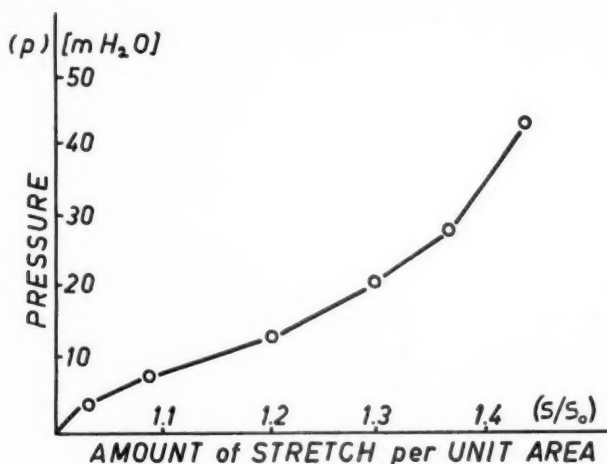


Fig. 4. — Area-pressure curves of normal fresh frog skin preparations.

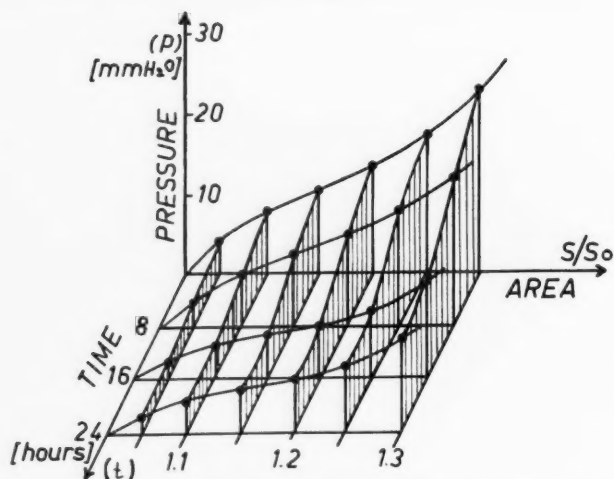


Fig. 5. — The change in the area-pressure curve with the age of the preparation.

dimensional, with the area distension ( $S/S_0$ ) and the time ( $t$ ) as horizontal plane coordinates and the pressure as the vertical co-ordinate. The slope of the curves diminishes with the time.

In Fig. 6 two curves are shown, one of the ventral and one of the dorsal skin preparations. It can be seen that the skin of the ventral region gives an area-pressure curve with a steeper slope than the dorsal region skin. In this figure an area-pressure curve of rubber is also given. The slope of this curve is steeper than those of the

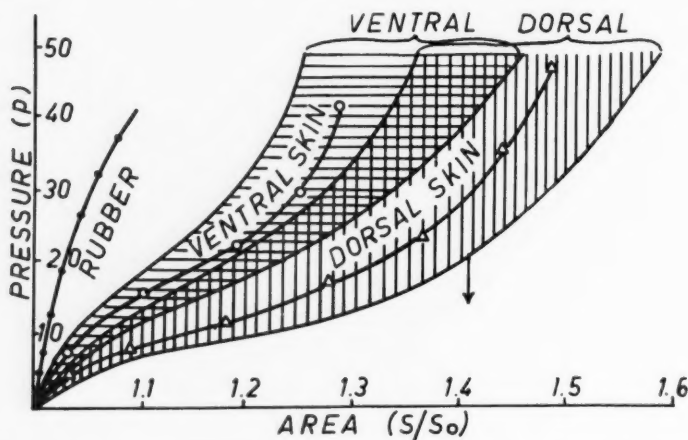


Fig. 6. — Area-pressure curves of rubber, ventral region and dorsal region skin preparations. The shadowed areas are the variations of different preparations.



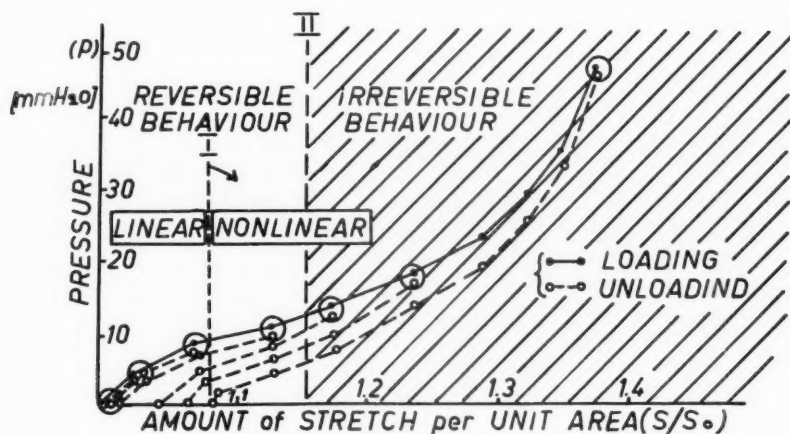


Fig. 7. — Unloading experiments with frog skin preparation.

skin preparations. The shadowed areas give the limits within which varied the results of the skin preparations from different frogs.

Fig. 7 shows the results of unloading experiments. The continuous curve (dorsal skin) shows the area-pressure relation in loading and the interrupted curves in unloading. The unloading was carried out from different degrees of stretch, and it was found that the stress-strain relation was linear and reversible below a certain amount of stretch (vertical dotted line I), nonlinear and reversible

TABLE 1  
AREA-PRESSURE RELATION OF FROG SKIN AND RUBBER (IN MM H<sub>2</sub>O/CM<sup>2</sup>).

|           | Pressure (p)<br>(mm H <sub>2</sub> O) | Area (S)<br>(cm <sup>2</sup> ) | Distension (S/S <sub>0</sub> )<br>(per unit area) | Elasticity (p/S)<br>(mm H <sub>2</sub> O/cm <sup>2</sup> ) |
|-----------|---------------------------------------|--------------------------------|---|--|
| Frog Skin | 5,9                                   | 4,70                           | 1,020   | 1,26   |
|           | 6,0                                   | 4,80                           | 1,045   | 1,25   |
|           | 10,5                                  | 5,30                           | 1,153   | 1,98   |
|           | 18,0                                  | 5,90                           | 1,282   | 3,06   |
|           | 28,0                                  | 6,42                           | 1,391   | 4,38   |
|           | 42,1                                  | 6,70                           | 1,458   | 6,29   |
| Rubber    | 15                                    | 4,70                           | 1,020   | 3,19   |
|           | 29                                    | 4,80                           | 1,065   | 6,04   |
|           | 49                                    | 5,30                           | 1,153   | 9,25   |
|           | —                                     | 5,90                           | 1,282   | —  |
|           | —                                     | 6,42                           | 1,391   | —  |
|           | —                                     | 6,70                           | 1,458   | —  |

below a higher degree of stretch (vertical dotted line II) and non-linear and irreversible above this latter degree of stretch (the shadowed area). These results correspond to the whole material. Especially the limit of the linear relation showed a great variation (arrow in Fig. 7).

In table I are given the experimental values of the pressure ( $p$  in mm H<sub>2</sub>O), area ( $S$  in cm<sup>2</sup>), distension per unit area ( $S/S_0$ ) and elasticity ( $p/S$  in mm H<sub>2</sub>O/cm<sup>2</sup>) for a typical, normal frog skin preparation, and for a circular rubber strip of approximately the same thickness as the skin. It can be seen that the value ( $p/S$ ) increases, after an initial slight decrease, by increase of stretch in the skin preparation.

#### DISCUSSION

By means of the method used it was possible to show the stress-strain relation in frog skin tissue. This relation was found to give a curve like those obtained with several organic tissues, *i.e.* muscle (2), skin (9), bladder (7), aorta (4) etc. In comparison with rubber, the amount of stretch corresponding to a certain value of pressure was greater. A linear, reversible behaviour in the area-pressure relationship was observed only in slight distensions (approx. to the value 1.1 of the amount of stretched area per unit area). The reversible extensibility ranged to a larger amount of the stretch (to the value 1.2 of the amount of stretched area per unit area). Stretching more, the behaviour was reversible only after a sufficiently long period of rest. It is concluded that the stress-strain relation in the frog skin is highly timedependent in that the skin tissue takes its original *in situ* shape only after a longer period of rest. However, these time-dependent relations belong to the phenomenon of plasticity and were not the object of this study. When left in Ringers solution for 24 hours, the increase of the area of the skin, which was caused by an increased pressure, was more rapid than in fresh preparations. The skin preparations of the dorsal body surface were found to be more extensible with a given value of the pressure than the skin preparations of the ventral surface.

## SUMMARY

The stress-strain relations of the frog skin (*Rana temporaria*) were studied. The relation was found to give area-pressure curves like those obtained with several organic tissues. A linear, reversible behaviour was observed only in slight distensions of about 10% of the undistended area.

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## THE PARALYTIC/INFECTED RATIO IN A SUSCEPTIBLE POPULATION DURING A POLIO TYPE I EPIDEMIC

by

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With the technical assistance of Miss DYVEKE BREMER

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This paper is based on the results obtained during the field trial of poliomyelitis vaccine in 1954 in Finland. Because the antigenicity of the vaccine used in 1954 was poor and lacking of type I antigen it was possible to follow relatively accurately the serologic conversion rate against polio virus type I during the heavy polio epidemic in the autumn of 1954. When the serologic and clinical data were compared, statistical evidence was gained which favoured the hypothesis that the incidence of paralysis among the cases of infection increased in the course of this epidemic.

### METHODS

The set up of the field trial in Finland was similar in principle to that of placebo controlled trial in the U.S.A. (1). The vaccine-placebo code scheme, however, was simplified so that only two codes were used, AA and BB. Thus labelled, the packages were distributed equally to each vaccine clinic. The only difference between vaccine

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and placebo noted by the nurses who were mainly responsible for the injections was that one type (AA) foamed somewhat more when shaken than the other (BB). The age range of the children (c. 20,000) who participated in the study was 6—12 years.

The first blood specimen was collected in June before the first intramuscular injection, the second in August two-three weeks after the third injection, and the third specimen in December about six months after the first.

The existence or non-existence in sera of antibodies against different types of polio was determined at that time in our laboratory by neutralization tests in HeLa cell cultures. There was no incubation of the virus and serum mixture before the test proper. The virus strains were Brunhilde, Lansing and Saukett. The quantity of used virus in the test was of the order of 30 TCD 50. Serum controls and virus titration were always included in the neutralization series. The final dilution of the serum in the test was 1/8. In investigating the change in successive serum specimens the sera were tested in the *same* neutralization test series and only a change from 4 + tissue destruction to complete protection was accepted as a sign of antibody production. Such a change occurred when the same serum was retested in *different* neutralization test series with 3 out of 118 sera. A part of the serological survey was carried out in Pittsburgh by the courtesy of Dr Salk (cf. below).

#### RESULTS

*Serologic Data.* — The immunity status of the population in question before the first injection is presented in Table I. The vaccine (A) and placebo (B) groups are presented separately to permit an estimate of the statistical reliability of the results.

TABLE 1  
ANTIBODY STATUS AGAINST THREE TYPES OF POLIO VIRUS BEFORE THE VACCINATION

| Group | Type I |     |                    | Type II |     |                    | Type III |     |                    |
|-------|--------|-----|--------------------|---------|-----|--------------------|----------|-----|--------------------|
|       | Number |     | %<br>Posi-<br>tive | Number  |     | %<br>Posi-<br>tive | Number   |     | %<br>Posi-<br>tive |
|       | +      | —   |                    | +       | —   |                    | +        | —   |                    |
| A     | 293    | 170 | 63                 | 177     | 249 | 42                 | 274      | 171 | 62                 |
| B     | 252    | 160 | 61                 | 175     | 202 | 46                 | 239      | 146 | 62                 |

According to Table I, more than 60 per cent of the children had antibodies against types I and III but less than 50 per cent against type II. Groups A and B show no statistical differences, as was to be expected.

The serological effect of three injections of the experimental Salk vaccine lot used in Finland can be seen from Table II.

TABLE 2

THE INCREASE IN NUMBER OF POSITIVE REACTORS AFTER THREE INJECTIONS OF EXPERIMENTAL SALK VACCINE (A) AND PLACEBO (B)

| Group | Type I |    |            | Type II |    |            | Type III |    |            |
|-------|--------|----|------------|---------|----|------------|----------|----|------------|
|       | Number |    | % Positive | Number  |    | % Positive | Number   |    | % Positive |
|       | Tested | +  |            | Tested  | +  |            | Tested   | +  |            |
| A     | 134    | 16 | 12         | 92      | 31 | 34         | 63       | 16 | 25         |
| B     | 147    | 16 | 11         | 85      | 1  | 1          | 55       | 2  | 4          |

Table II presents the results obtained when negatives in the first specimen were tested again in the same neutralization test series together with the second specimen. Only the change from 4+ tissue destruction in the first serum specimen to complete protection in the second serum was accepted. Table II shows that the differences between the vaccine and placebo groups are highly significant for types II and III but that there was no difference as regards type I. The placebo thus had a great value in this study and erroneous conclusions would have been reached without it, especially if the changes in the third specimen referred to later, had also been counted. The most reasonable explanation for the increase in the number of positive reactors against type I

TABLE 3

THE INCREASE IN POSITIVE REACTORS AGAINST TYPE I VIRUS AFTER VACCINATION WITH EXPERIMENTAL SALK VACCINE. RESULTS IN TWO DIFFERENT LABORATORIES (PITTSBURGH AND HELSINKI) SEPARATELY. SPECIMENS TESTED IN THESE TWO LABORATORIES COME FROM DIFFERENT AREAS

| Group | Pittsburgh |   |            | Helsinki |    |            |
|-------|------------|---|------------|----------|----|------------|
|       | Number     |   | % Positive | Number   |    | % Positive |
|       | Tested     | + |            | Tested   | +  |            |
| A     | 59         | 4 | 7          | 75       | 12 | 16         |
| B     | 52         | 3 | 6          | 95       | 13 | 14         |

in both groups is the starting polio epidemic. The ineffectiveness of the vaccine used as regards type I is further confirmed in Table III where the results of two different laboratories are given separately.

Serologic conversion rate in the two laboratories is different because the specimens come from different areas. Some idea of the sensitivity of the methods in the two laboratories can be obtained from the following figures: When specimens from the city of Helsinki were investigated in Helsinki 310 of 453 specimens had type I antibodies *i.e.* 68 per cent; the corresponding figures in Pittsburgh were 33 of 54 *i.e.* 61 per cent.

The overall effect of the epidemic on type I antibodies is illustrated in Table IV.

TABLE 4

THE INCREASE IN NUMBER OF POSITIVE REACTORS AGAINST TYPE I VIRUS DURING THE POLIO EPIDEMIC. ALL THREE BLOOD SPECIMENS OF THE CASES NEGATIVE IN THE FIRST SPECIMEN WERE TESTED IN THE SAME NEUTRALIZATION TEST SERIES

| Number of Blood Specimen | Group A |    |            | Group B |    |            |
|--------------------------|---------|----|------------|---------|----|------------|
|                          | Number  |    | % Positive | Number  |    | % Positive |
|                          | Tested  | +  |            | Tested  | +  |            |
| 2.                       | 134     | 16 | 12         | 147     | 16 | 11         |
| 3.                       | 126     | 34 | 27         | 126     | 31 | 25         |

It can be seen from Table IV that the vaccine and placebo groups behave very similarly, a point which was observed in Table II. The result indicates that *c.* 25 per cent of the persons in the field trial age groups and areas who did not have antibodies before the epidemic developed them during the epidemic.

The changes in the numbers of positive reactors against type II and III polio virus can be seen in Table V and VI.

TABLE 5

THE CHANGES IN THE NUMBER OF POSITIVE REACTORS AGAINST TYPE II VIRUS

| Number of Blood Specimen | Group A |    |            | Group B |   |            |
|--------------------------|---------|----|------------|---------|---|------------|
|                          | Number  |    | % Positive | Number  |   | % Positive |
|                          | Tested  | +  |            | Tested  | + |            |
| 2.                       | 92      | 31 | 34         | 85      | 1 | 1          |
| 3.                       | 85      | 21 | 25         | 71      | 9 | 13         |



TABLE 6

THE CHANGES IN THE NUMBER OF POSITIVE REACTORS AGAINST TYPE III VIRUS

| Number of Blood Specimen | Group A |    |            | Group B |   |            |
|--------------------------|---------|----|------------|---------|---|------------|
|                          | Number  |    | % Positive | Number  |   | % Positive |
|                          | Tested  | +  |            | Tested  | + |            |
| 2.                       | 63      | 16 | 25         | 55      | 2 | 4          |
| 3.                       | 55      | 15 | 27         | 44      | 2 | 5          |

It can be seen from Table VI that the antibody status as regards type III did not change during the polio epidemic and that the effect of vaccination as regards antibodies produced remained unchanged 3—4 months.

Before examining the results for type II presented in Table V, it must be mentioned that although the epidemic of fall 1954 was caused by type I, judging by numerous virus isolations from the patients, one strain of type II also was isolated from a non-paralytic case (2). The significant increase of antibodies in the placebo group is probably due to type II infections. The non-significant changes in the vaccine group, if any conclusions can be drawn from them, might be due to the weakness of the antigen used and the brief antibody response caused by it.

*Change in the Paralytic/Infected Ratio.* — The data presented above were analysed for a possible change in the ratio of paralytic to infected cases during the first and second period of the epidemic divided according to the collection of specimens. The date dividing the periods was set at August 31 although the majority (88 per cent) of the second blood specimens (763) were collected between August 1 and 23. It is assumed that poliomyelitis virus type II has not produced many paralytic cases. This is supported by the virus isolations from paralytic cases. From 116 subjects type I virus was recovered in 64 and type II in one case (2). The paralytic cases were divided according to the date of the first reported signs of the disease. The study population, according to official statistics, was very near 100,000. Table I shows that c. 38 per cent of the study population did not have antibodies against polio type I. Thus it can be considered that c. 38,000 children

TABLE 7

RATIO OF PARALYTIC CASES TO INFECTED CASES IN THE FIRST AND SECOND PERIOD OF THE EPIDEMIC.

| Time Between     | Cases of Paralytic Poliomyelitis (P) | Infected Cases (I) | P/I   |
|------------------|--------------------------------------|--------------------|-------|
| Specimen 1 and 2 | 17                                   | 4,200              | 1/250 |
| Specimen 2 and 3 | 47                                   | 5,300              | 1/110 |
| Specimen 1 and 3 | 64                                   | 9,500              | 1/150 |

were susceptible. The calculations presented in Table VII have been made as regards to this »susceptible» part of children.

According to statistical analysis it would be possible to maintain that the rate P/I changed between the first and second period of the epidemic.

## GENERAL DISCUSSION AND SUMMARY

The data presented in this paper are derived from the poliomyelitis field trial. They might, however, have some interest as regards unapparent infection rate, value of placebo technique and increase in incidence of paralysis in the course of the epidemic. During experimentation with the live polio vaccine the possibility of a change in the ratio of paralytic to infected cases during epidemic spread of virus in the community is of considerable interest.

The antigenic insufficiency at that time of the killed polio-virus vaccines emerges clearly from the data. The vaccine lot used in the field trial, No 513, was considered to be moderately good although relatively less useful against type I (1).

The results show clearly the value of the placebo technique. Without it completely misleading results would have been gained as regards the effectivity of the type I component. The special circumstance observed in this case — complete ineffectiveness of the type I component simultaneously with a type I epidemic — made it possible to estimate with considerable reliability the rate of unapparent infection. With results from one group only the explanation of the serologic conversions would have been more difficult and not so significant. According to repeated titrations in different series, the technical error was under 3 per cent. When

the sera for comparison from the same person are practically always titrated in the same neutralization series, the laboratory error should be even lower. The part played by technical error in the present study can thus be estimated as small.

The similarity of the results in the vaccine and placebo group (Tables II and IV) excludes the possibility of antibody production against Type I by other vaccine components. The stable antibody status against type III virus after the initial increase caused by the vaccine can be considered an additional proof of the reliability of the results. There were no indications of a type III virus spread in Finland in the fall of 1954, unlike the case with types I and II (2). It is difficult to escape the conclusion that the measured antibody increase against type I in the population was due to unapparent infection. According to this study, the unapparent infection rate during the type I epidemic was close to 25 per cent in the «susceptible» population.

Some of the results published in the Final Report of Field Trial (1) are of interest in this connection. These concerned the serological investigations reported by single laboratories and thus without inter-laboratory variations, which made it difficult in general to estimate the unapparent infection rate. For example, in Shelby County, Tennessee, the percentage of reported serologic conversions in «non-case» controls for type III was 27, in Jefferson County, Kentucky, 26 per cent and in Harris County, Texas, for type I 17 per cent. The percentages above are in good conformity with the results presented in this study.

Comparison of the results from Pittsburgh and Helsinki (Table III) suggest that the laboratory technique in Helsinki was not less sensitive. The relatively high final concentration (1/8) of the serum to be investigated in the neutralization test may have been of importance and possibly diminished the effect of the lack of pre-incubation of serum and virus mixture.

The considerations governing the P/I ratios in Table VII have been already presented above. The calculations were made with caution and as regards the dividing date for the two periods, biased against the hypothesis of a possible change in P/I ratio. The difference between the initial and later period, 1/250 against 1/110, is of an order that merits attention. If the observed difference is real it can be caused by a change in the behaviour

of the virus or by other conditions such as the appearance of other »supporting» viruses. Studies with live virus vaccines show that polio virus may change its paralysis-producing property (3, 4). It is a short step from this to the assumption that the results presented are due to this change during the natural dissemination of the polio virus.

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## STUDIES IN THE ELECTRICAL BEHAVIOUR OF THE RESTING CELL MEMBRANE OF *NITELLOPSIS OBTUSA*<sup>1</sup>

by

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There is definite interest in studying the electrical phenomena of some plant cells for comparison with the similar processes of animal cells. With a view to studying the effects of ionizing radiation on the electrical functions of cell membranes, a species of the alga Characae, *Nitellopsis obtusa*, was selected as the object. The large cells of this brackish water alga measure up to 10 cm in length and 0.8 mm in diameter, and show a considerable resistance to mechanical disturbances, as compared with some other species of this family. They thus form a suitable object for handling in experiments, for the insertion of electrodes etc.

The cells of *Nitellopsis obtusa* and related species show a large central vacuole filled with sap, a thick cellulose wall and an adjacent protoplasmic layer. This layer is limited to the sap with the aim of a membrane, known as the tonoplast. There is also assumed to be a membrane on the outer border of the protoplasm close to the wall of the cell. This latter membrane is thought to be polarized, so that the protoplasm shows a negative potential in relation to the natural environment of the cell. This potential is of considerable magnitude, about 100–200 mV (1, 2). The membrane, assumed to consist of lipoproteins (3), shows a characteristic behaviour of permeability to both non-electrolytes (4) and ionized particles of electrolytes (2, 5), the resting potential resulting from a selectivity in the

<sup>1</sup> This work was supported by a grant from The Finnish National Committee for Natural Sciences.

permeation of the ions. McRobbie and Dainty (2), who studied in tracer experiments the rates of exchange of the ions of sodium, potassium and chloride in single cells of *Nitellopsis obtusa*, found a high K/Na ratio of the cell interior. The authors suggested an active outward sodium transport at the outer protoplasmic membrane of the resting cell, which would maintain the resting potential of the membrane (120—200 mV).

This study is concerned mainly with measurement of the resting membrane potential of internodal cells of *Nitellopsis obtusa* over a period of 2—3 days. A method was thus developed in which the cells could during this period be kept under physiological conditions in order to study the magnitude and constancy of this potential.

#### METHOD

In comparison with other characeans *Nitellopsis obtusa* appears to be very rare. In Finland, it can so far be obtained only in limited regions near Porvoo and in Snappertuna, where it grows in the brackish sea water at a depth of about 2 m. The plants can easily be transported without damage if sealed in plastic material to prevent them from drying (the cells studied by McRobbie and Dainty (2) were from Finland). The cells used in this study were collected from Snappertuna, and stored in artificially brackish water similar to the normal environment. The original seawater from Snappertuna was analysed, and on the basis of the finding a solution of the following composition was prepared:

|                          |       |           |   |
|--------------------------|-------|-----------|---|
| NaCl .....               | 2.149 | per mille |   |
| CaCl <sub>2</sub> .....  | 0.130 | »         | » |
| KCl .....                | 0.052 | »         | » |
| MgCl <sub>2</sub> .....  | 0.174 | »         | » |
| MgSO <sub>4</sub> .....  | 0.270 | »         | » |
| NaHCO <sub>3</sub> ..... | 0.084 | »         | » |

In view of the aim of the study, that of obtaining a stable culture of the plants, some cell material was also stored in the artificial solution, to which pure garden soil had been added. In fact, this excess of organic materials appeared to be useful, as in the culture the growth of new cells was more rapid as far as this material was concerned. The cells used in this study were, however, from the first mentioned culture.

The plants were stored in a chamber with weak illumination and a temperature of approx. 18°C. The solution was aerated. Under these conditions, the plants seemed to grow well. The storage was continued for a minimum of one month before experiments were begun.

Large sized internodal cells were dissected for the study, and kept dissected in the artificial solution for one week before the experiment. Two types of cells were used, young cells situated near the growing end cells, and older cells with a darker brownish colour. Colourless »white« cells, which appeared to be very common (the plants were collected in October) and seemed to be fresh and undamaged, were not used in this study.

In order to measure the resting potential of the cell membrane, microelectrodes were used so as to keep the cells in their natural environment, and to prevent damage to the cell. The glass canyles (Pyrex, tip diameter appr. 10  $\mu$ ) were filled, by boiling at reduced pressure, with 0.2 M KCl, corresponding to the mean Cl concentration of the sap (2). Silver wires coated electrolytically with silver chloride were used in conjunction with the microelectrodes. Such silver wires alone served as external electrodes. Since the electrode potentials of the two electrodes could not be the same, the difference between them was controlled before and after each experiment (in some instances, several times during the course of the experiment). This potential difference was taken into account in evaluating the resting membrane potential.

The general experimental arrangement is shown in Fig. 1. The plant cell was kept in a glass capillary container in the artificial storage solution. The AgAgCl electrode was immersed in this solution; this served as the external electrode. The solution was changed with a sufficient volume of fresh aerated solution during the course of the experiment by means of a thin capillary. The system could be kept closed, as the microelectrode was pressed against the glass capillary serving as container for the plant cell. To prevent leakage between the cell content and the surrounding fluid, a part of the neighbouring cell was left connected with the cell to be studied. This part could be pressed between the microelectrode and the container.

The electrodes were connected to a D.C. amplifier (GR) with an input resistance of  $10^{14}$  ohm. The records were made with an Ester-



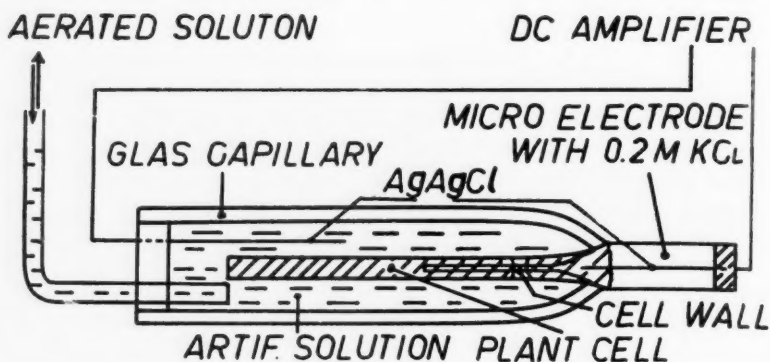


Fig. 1. — Schematic representation of the experimental conditions (see Method).

line-Angus recorder, and could thus be followed continuously for several days. The temperature and the illumination were the same as during the culturing of the plants.

About 40 internodal cells of *Nitellopsis obtusa* were studied under exactly the same conditions as above, but recordings of resting membrane potentials were made by slightly different methods, with ionizing radiation, with more than one hundred cells during the course of the experiments. About 25 per cent of those studied were of the «old cell» type.

#### RESULTS

In view of the values of the resting membrane potentials for the two types of cells studied, it was found that older cells with a brownish color mainly showed a lower membrane potential than the younger cells with the light green colour. The potential of the former type ranged between 40 and 100 mV inside negative (9 cells studied), whereas the potential of the latter type was between 70 and 160 mV inside negative (28 cells studied). The distribution of the resting potential values obtained in the series is shown in Fig. 2. No grouping of the value could be found around a mean. All these values were recorded initially after a stable state of the record had been reached.

Despite the great variability of the resting potential from cell to cell, a considerable constancy of the recorded potential in the



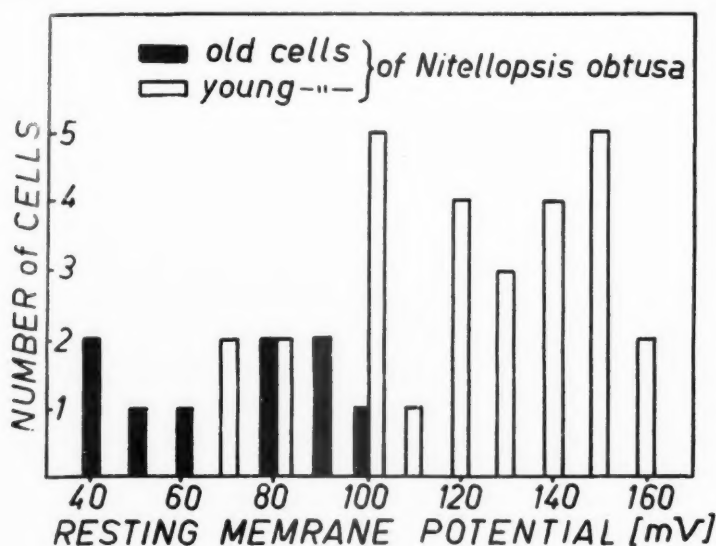


Fig. 2. — The distribution of recorded resting membrane potential values in a material of 37 internodal cells of *Nitellopsis obtusa* (white: young, black: old cells).

same cell could be observed during the whole time of observation. This time ranged in most of the experiments up to 40 hours, but in some cases the potential could be followed for as long as 70 hours. The experiments were mainly interrupted because of external accidents due to renewed punctures of the cells (in control of the electrode potential). It could be noted that the potential measured under the given conditions dropped by approximately 10 per cent during the first 24 hours, and slightly more during the other periods of 24 hours. In this respect the drift of the recording instruments themselves was taken into account. A checking of the membrane potential on three successive days in a typical experiment is shown in Fig. 3. In this case the initial potential was 105 mV at the beginning of the experiment. The decrease observed during three days was approx. 15 per cent. The lowering of the potential can result either from the disturbances resulting from the renewal of the surrounding solution, or from a leakage of the cell content due to the micro-electrode.

Only slight oscillatory fluctuations of the resting membrane potential in some cells were observed. These fluctuations ranged up

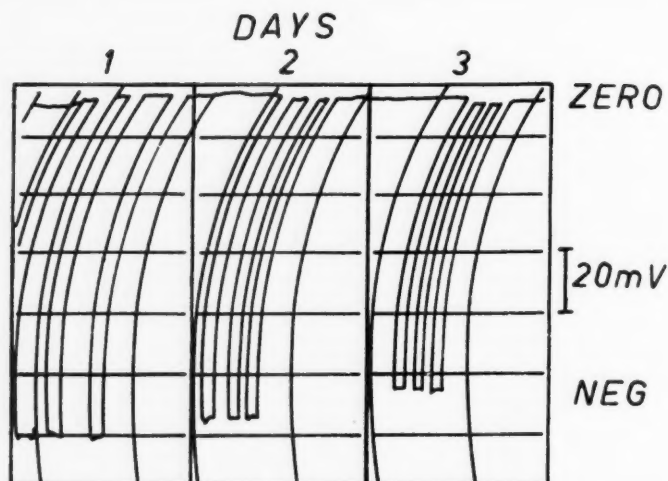


Fig. 3. — Resting membrane potential recordings from one and the same cell under the same physiological conditions during an experiment (the base line, 'zero', above). Note the slight decrease of the potential due to the experimental conditions.

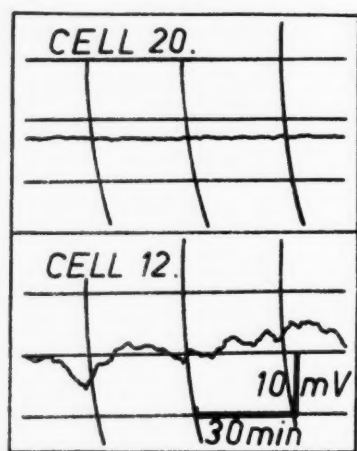


Fig. 4. — Two tracings of the resting membrane potential in *Nitellopsis obtusa*, made from two cells with different behaviour. The lower shows oscillations (typical of the cell in question) with an amplitude of about 10 mV and a period of 10 min.

to about 5 to 10 per cent of the resting value, and consisted of slow oscillations with a period of 2 to 10 min. Their origin is obscure. When these oscillations occurred, they seemed to be typical of the cell concerned, and appeared in rhythms after silent periods which

could last for hours. In most of the cells, however, a steady potential was measured during the whole period of recording, *i.e.* for 2—3 days (with the slow decrease mentioned above). In Fig. 4 are two tracings the upper from a «silent» cell, and the lower from a cell showing small oscillations of the resting potential.

#### DISCUSSION

The experiments show that the brackish water alga *Nitellopsis obtusa* can quite well be cultured under laboratory conditions in artificial solutions (see Method). It is accordingly especially suited for studies of the physiological functions of living cell membranes (2, 5) and other cell structures.

The measurements showed that the resting membrane potential of the internodal cells of *Nitellopsis obtusa* varies within large limits from one cell to the other (measured values 40 to 160 mV inside negative), but shows considerable constancy in one and the same cell. The values of the recorded potentials are lower than those reported by McRobbie and Dainty (2). In this material, and in subsequent experiments using different methods, potentials could on no occasion be observed which ranged over the above values.

In the older cells of brownish colour, the potential could be as small as 40 mV, possibly due to a slower metabolism in older cells.

The experiments also show that the method represented above provides a possibility of studying the cell processes during a period of several days with the cells stored under physiological conditions.

#### SUMMARY

Large cells of the brackish water alga *Nitellopsis obtusa* were stored in artificial solutions under laboratory conditions; here they seemed to grow well. A method was developed in which the resting membrane potential could be measured with microelectrodes for a period of three days without interruption, with the cells under physiological conditions. The resting potential varied from 40 to 160 mV (inside negative) in different cells, being lower in older cells than in younger. In the same cell, however, the potential was constant with only small oscillations (amplitude 5 to 10 per cent

of the total membrane potential, and period 2—10 min.). Such oscillations occurred in only few cases, and were typical of the cells concerned during the time of observation.

My thanks are due to Professor R. Collander, Dr. Phil., and Docent V. Wartiovaara, Dr. Phil., for their kind help in obtaining the plant material used in this work.

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## THE EFFECT OF 5.3 MeV POLONIUM<sup>210</sup> ALPHA-PARTICLES ON THE ELECTRICAL ACTIVITY OF $\alpha$ - AND $\beta$ -FIBRES OF THE FROG SCIATIC NERVE

by

R. M. BERGSTRÖM

(Received for publication April 2, 1961)

In an earlier work (5) some effects of Polonium<sup>210</sup> alpha-particles on the electrical activity of single nerve fibres of frog were described. It appeared that relatively small doses of these heavy ionizing particles are needed to produce changes of the action potential when the irradiation is performed in a circumscribed manner from a ring-shaped Po<sup>210</sup> source (4, 6). It could also be observed in accordance with the findings of Bachofer (1) that ionizing radiation produces an initial enhancement of the activity of the nerve, which is followed by a block in the conductivity. These alterations of the nervous activity due to the irradiation seem to be independent of the type of the ionizing radiation.

During the work on single nerve fibres of the frog, some preparations exhibited electrical signs of different types of fibre. Thus bundles containing  $\alpha$ - and  $\beta$ -fibres could be obtained relatively easily from the sciatic nerve. The question then arose as to the manner in which the alpha-irradiation affects the electrical activity of these different types of fibres.

### METHOD

As regards the method of irradiation, reference is made to the above work (6). The nerve bundles were excised from the sciatic nerves of decerebrated frogs (*Rana temporaria*) and prepared in

oxygenated Tasakis Ringer solution (8) at 18°C (the temperature employed in the following experiments). The bundles were tested with electrical stimulation in order to obtain a compound spike configuration which indicated the presence of  $\alpha$ - and  $\beta$ -fibres (7). Such bundles were irradiated with alpha-particles and the action potentials brought about by the stimulation with square wave pulses recorded partly during and partly after the irradiation. The irradiation doses varied from 200 to 10000 rep, the dose rate being 800–1400 rep/min. The registration of the action potentials was made with AgAgCl-electrodes, differential preamplifier (Tönnies) and cathode-ray oscillograph (Tektronix 502) on standing film. After the experiment, the bundles were examined histologically. Only bundles with a diameter less than 40  $\mu$  were treated in the material to ensure that all fibres in it were penetrated by the alpha-particles. Altogether, 11 such bundles were studied.

#### RESULTS

Fig. 1 shows a typical record of the action potential of a bundle with histologically 5 intact fibres before and after irradiation with  $\text{Po}^{210}$  alpha-particles (dose 6000 rep, 1200 rep/min). It can be seen that the greater  $\alpha$ -potential decreases to a value of approx. 50 per

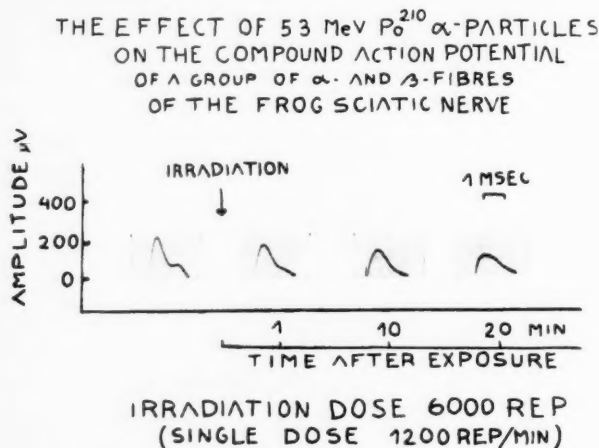


Fig. 1. — A typical record of the action potential of a preparation of the sciatic nerve of the frog (5 intact fibres) before and after irradiation with 5.3 MeV  $\text{Po}^{210}$  alpha-particles. Dose 6 000 rep (1200 rep/min).

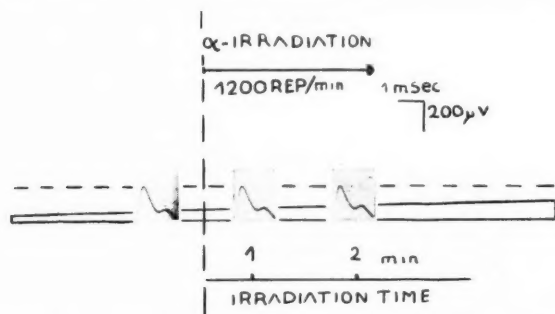


Fig. 2. — Immediate changes of the compound action potential of a fibre preparation (6 intact fibres) of the frog sciatic nerve during irradiation with 5.3 MeV  $\text{Po}^{210}$  alpha-particles.

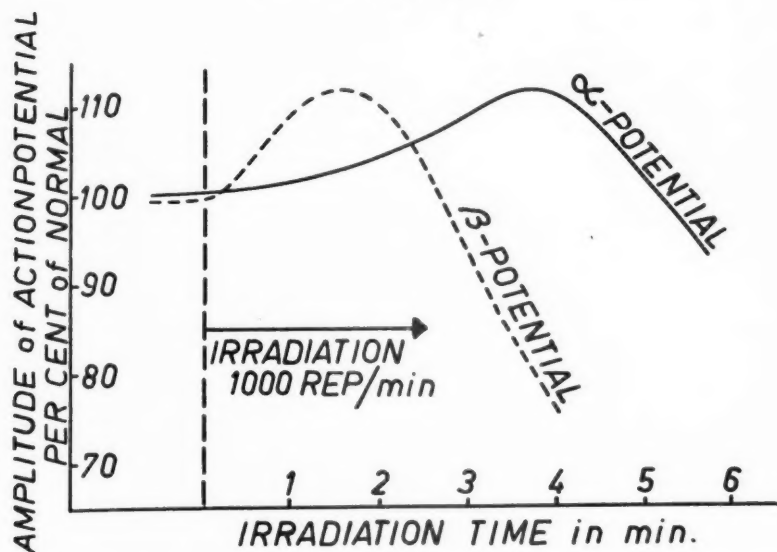


Fig. 3. — Typical behaviour of the amplitude of the  $\alpha$ - and  $\beta$ -elevations of the compound action potential of fibre bundles of the frog sciatic nerve after the beginning of the irradiation with alpha-particles. The dose rate approx. 1 000 rep/min.

cent in a period of 20 min after the irradiation. The  $\beta$ -elevation of the compound potential disappears completely in 10 min.

The initial increase of the action potential due to the alpha-irradiation, as reported in the work referred to above, was also observed in these experiments, especially with irradiation doses smaller than 6000 rep. However, this increase in the action potential had a different time course for the  $\alpha$ - and the  $\beta$ -elevation of the

compound potential. Fig. 2 shows the immediate changes of the compound potential of one fibre preparation (with histologically 6 intact fibres) after the beginning of the irradiation. In this case, the dose rate was similar to that shown in Fig. 1. It can be seen that the  $\beta$ -potential increases slightly during the first 2 min, whereas there cannot be observed any detectable change in the  $\alpha$ -potential.

Fig. 3 shows the typical behaviour of the amplitude of the  $\alpha$ - and  $\beta$ -elevations of the compound potential after the beginning of the irradiation with alpha-particles. In these experiments the dose rate of the irradiation was approx. 1000 rep/min. The maximum of the  $\beta$ -potential was attained in about 2 min, following which this potential declined rapidly. The  $\alpha$ -potential reached its maximum in about 4 min, and began to decrease more slowly than the  $\beta$ -potential.

#### DISCUSSION

The results indicate that the  $\alpha$ - and the  $\beta$ -fibres of the sciatic nerve of the frog show a different response to irradiation with heavy nuclear particles (5.3 MeV  $\text{Po}^{210}$  alpha-particles). Both types of fibres, as studied from the compound potential of small fibre bundles, showed an enhancement of the electrical activity, similar to that reported by Bachofer (1) and Bachofer and Gautereaux (2, 3) after X-Ray irradiation of *Lumbricus* giant fibres and mammalian nerves. The  $\beta$ -fibres, however, seemed to be more sensitive to the irradiation in that the enhancement of the  $\beta$ -potential appeared earlier than the enhancement of the  $\alpha$ -potential. Also the rate of diminution of the  $\beta$ -elevation of the compound potential was faster than that of the  $\alpha$ -elevation. Nevertheless it is difficult to know the absolute changes of the action potential of the  $\beta$ -fibres, since this potential overlaps the after potentials of the  $\alpha$ -fibres. The possibility that the reported initial increase of the  $\beta$ -potential could result from a change of the later parts of the  $\alpha$ -potential, is uncertain, as the results of an earlier study (5) with single fibres indicate that the shape and magnitude of a typical  $\alpha$ -fibre does not change with irradiation doses as small as those used here.



## SUMMARY

Irradiation of small bundles of frog sciatic nerve with 5.3 MeV Polonium<sup>210</sup> alpha-particles results in an initial enhancement of the  $\alpha$ - and  $\beta$ -potentials of the compound action potential. This enhancement, preceding the blocking of the conductivity of the fibres, appears earlier in the  $\beta$ - than in  $\alpha$ -fibres. The decline of the potential due to the irradiation occurs in the  $\beta$ -fibres at a faster rate than in the  $\alpha$ -fibres.

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## TRANSFORMATION OF BIOELECTRIC POTENTIALS TO SQUARE WAVE PULSES SUITABLE FOR A PULSE COUNTER DEVICE

A SIMPLE METHOD UTILISING TEKTRONIX 502 DUAL BEAM  
OSCILLOSCOPE

by

R. M. BERGSTRÖM and R. W. F. BLÄFIELD

(Received for publication April 26, 1961)

The transformation of bioelectric potentials (EMG, EEG, nerve potentials etc.) to pulses of a uniform character which may be employed for the counting of the wave spikes or as trigger pulses, for various experimental purposes, requires special equipment. This paper reports on a simple method, utilising the Tektronix 502 dual beam oscilloscope to this end.

### METHOD

The built-in time-base trigger of the oscilloscope is used. This provides, in the ac mode stable triggering on virtually all types of wave-form. An external output connector is mounted to the panel, and this is connected through a capacitor to the anode (point 1) of the time-base output stage 6DJ8 (tube V45B). The specimen to be tested is connected through a biological amplifier to the trigger input, and to the amplifier input of one of the beams. This external trigger output is connected to the amplifier input of the other beam and may also be connected to an electronic counter. By this means, both traces, the original wave form and the squared pulses of equal amplitude, are visible on the face of the cathode-ray tube screen and the trigger level control may be adjusted to the desired point.

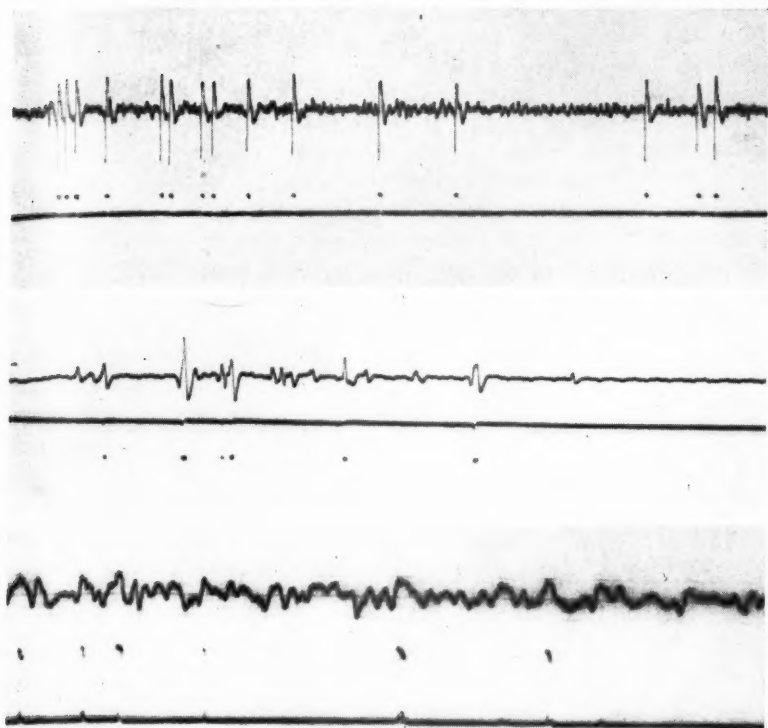


Fig. 1. — Potentials from single nerve fibres of frog skin nerve (above), human finger muscle and muscles of a worm (below) transformed to square wave pulses by means of a Tektronix 502 dual beam oscilloscope (see text).

Fig. 1 shows photographs obtained by this means from single nerve fibres of skin nerve of the frog (above), motor unit potentials from human finger muscles and a rather complicated wave-form from the muscles of a lower worm (*Gordius aquaticus*). The trigger level is adjusted in all three cases to select only the biological potentials of higher amplitude.

#### SUMMARY

A simple method is presented using Tektronix 502 dual beam oscilloscope for the transformation of bioelectric potentials to square wave pulses suitable for pulse counting, triggering etc.

## CONCENTRATION OF BLOOD GROUP ANTI-SERA

### A SIMPLE TECHNIQUE

by

JUSSI J. SAUKKONEN<sup>1</sup> and KARI CANTELL<sup>2</sup>

Received for publication March 27, 1961

The property of certain high molecular weight substances to strongly absorb water has been successfully utilized to concentrate biological fluids (cf. 1—3). For example, proteins of serum and cerebrospinal fluid have been concentrated by dialysis against solutions of gum arabic, dextran and polyvinylpyrrolidone. Only minor changes in the electrophoretic distribution of proteins have been detected after the concentration (3, 4). — These findings led us to investigate whether this method could be applied to «improve» weak blood group anti-sera. It will be shown that specific titers of certain blood group agglutinins could be raised by dialysing the sera against some colloidal solutions.

### MATERIAL AND METHODS

*Colloid Solutions.* — Polyvinylpyrrolidone (Kollidon 17) was a product of Badische Anilin- und Soda-Fabrik, A.G., Ludwigshafen/Rhein, West Germany. It will be called below PVP. Dextran (Macrodex) was obtained from Pharmacia, Uppsala, Sweden, and gum arabic from F.W. Berk et Co., London, Great Britain.

40% solutions of the substances were prepared in distilled water and the pH was adjusted to 7 by addition of concentrated sodium hydroxide. The solutions were stored at 0—5°C. Boiling and addition of penicillin and streptomycin have been used to keep the solutions sterile.

<sup>1</sup> Aided by grants from the Sigrid Jusélius and J. K. Paasikivi Foundations.

<sup>2</sup> Aided by a grant from the Sigrid Jusélius Foundation.

*Dialyzer Tubing.* — No. 4465-A2 of Arthur H. Thomas Co., Philadelphia, Pa., USA, was used.

*Sera and Titrations.* — Human «natural» and immune anti-A and anti-B sera were titrated at room temperature and human immune anti-D (Rh<sub>0</sub>) at 37°C. Dilutions were made in agglutination tubes with a Pasteur pipette. Equal amounts of a 3% red cell suspension were added and the results were read under microscope after 2 hours.

*Papain Treatment.* — One volume of packed cells was added to two volumes of 0.1% papain (The British Drug Houses Ltd., Poole, Great Britain) in 1/15 M phosphate buffer, pH 7.3, and the mixture was incubated at 37°C for 30 minutes. The cells were washed once with saline.

*Absorption of Sera.* — The human anti-D sera were absorbed once at room temperature with an equal volume of packed A<sub>1</sub> and/or B cells to remove isoagglutinins.

#### EXPERIMENTAL

The ability of the three substances to concentrate human serum was first studied. Five ml of serum was dialysed against a 20-fold volume of a colloidal solution. The decrease in the volume of serum as a function of time is shown in Fig. 1. It can be seen that all three substances concentrate serum at approximately the same rate. PVP was found to be best fitted for routine purposes. In addition, it was found that one batch of 40% PVP solution could be used up to 20 times without noticeable decrease in its concentrating capacity.

Typical findings of the effect of concentration on the isoagglutinin titers of human sera are given in Table 1. It can be seen that 4 to 6-fold concentration of sera gave a corresponding rise in the specific titers of anti-A and anti-B. When dialysis was continued, further increase in the titer was not always evident, and sometimes unspecific agglutination was observed. In addition, sera concentrated 8-fold are too viscous to be easily handled with a pipette. It was observed that the use of PVP less frequently caused unspecific reactions than the use of gum arabic. Different batches of gum arabic varied in this respect whereas PVP, being a synthetic product, gave well reproducible results. Further examination of section V, Table 1, shows that titration of antisera in the presence of concentrated *heterologous* sera did not raise the titers. This could be taken to show that the increase in titers after concentration of homologous sera is caused by a higher concentration of specific agglutinins.

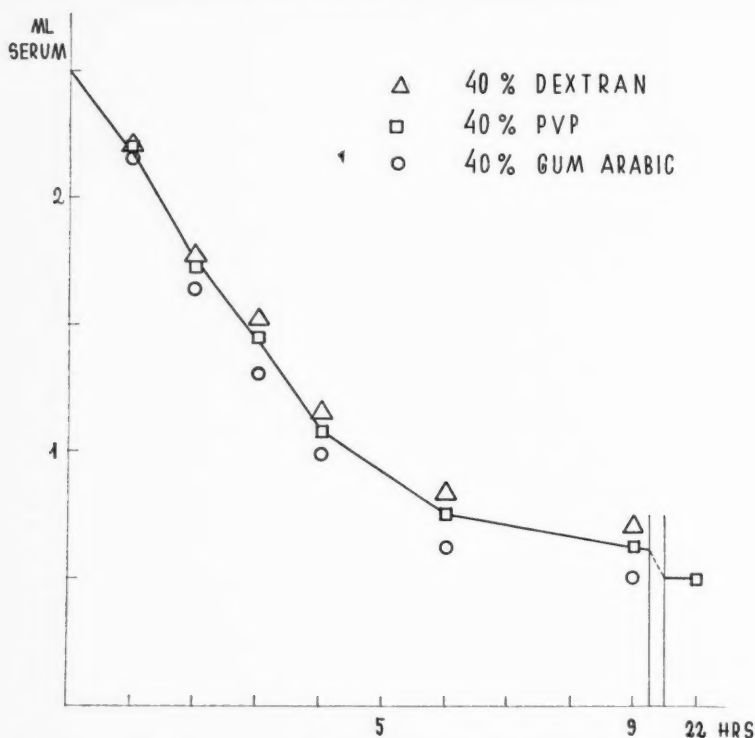


Fig. 1. — Rate of concentration of serum by the «standard method», using solutions of some high molecular weight substances.

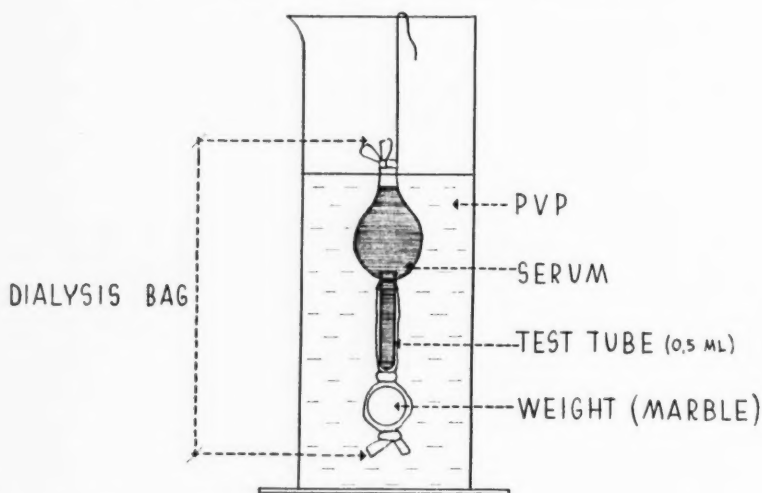


Fig. 2. — The «standard method» for obtaining a wanted degree of concentration.

TABLE 1  
CONCENTRATION OF ISOAGGLUTININS BY DIALYSIS AGAINST SOME HIGH MOLECULAR  
WEIGHT SUBSTANCES

| Substance   | Serum  | Reduction<br>in Serum<br>Volume | Diluent in<br>Titration | Titer          |   |   |   |    |     |    |     |     |     | Specificity <sup>1</sup> |    |   |
|-------------|--------|---------------------------------|-------------------------|----------------|---|---|---|----|-----|----|-----|-----|-----|--------------------------|----|---|
|             |        |                                 |                         | 1              | 2 | 4 | 8 | 16 | 32  | 64 | 128 | 256 | 512 | 1                        | 2  | 4 |
| I           |        |                                 |                         |                |   |   |   |    |     |    |     |     |     |                          |    |   |
| —           | anti-B | —                               | Saline                  | 4 <sup>2</sup> | 3 | 1 | — |    |     |    |     |     |     | —                        |    |   |
| PVP         | "      | 2-fold                          | "                       | 4              | 4 | 3 | 1 | —  |     |    |     |     |     | —                        |    |   |
| "           | "      | 4- "                            | "                       | 4              | 4 | 4 | 2 | —? | —   |    |     |     |     | —                        |    |   |
| "           | "      | 6- "                            | "                       | 4              | 4 | 4 | 3 | 2  | (+) | —  |     |     |     | —                        |    |   |
| "           | "      | 8- "                            | "                       | 4              | 4 | 4 | 3 | 2  | 1   | —  |     |     |     | 1                        | —  |   |
| II          |        |                                 |                         |                |   |   |   |    |     |    |     |     |     |                          |    |   |
| —           | anti-A | —                               | "                       | 4              | 4 | 4 | 4 | 4  | 3   | 1  | —   |     |     | —                        |    |   |
| Gum arabic  | "      | 4-fold                          | "                       | 4              | 4 | 4 | 4 | 4  | 4   | 3  | 2   | (+) | —   | —                        |    |   |
| "           | "      | 8- "                            | "                       | 4              | 4 | 4 | 4 | 4  | 4   | 3  | 2   | (+) | —   | 2                        | —? | — |
| III         |        |                                 |                         |                |   |   |   |    |     |    |     |     |     |                          |    |   |
| —           | anti-B | —                               | "                       | 4              | 4 | 3 | 3 | 2  | 1   | —  |     |     |     | —                        |    |   |
| Dextran     | "      | 4-fold                          | "                       | 4              | 4 | 4 | 4 | 3  | 3   | 2  | 1   | —   |     | —                        |    |   |
| "           | "      | 6- "                            | "                       | 4              | 4 | 4 | 4 | 3  | 3   | 1  | 1   | —   |     | —                        |    |   |
| IV          |        |                                 |                         |                |   |   |   |    |     |    |     |     |     |                          |    |   |
| —           | anti-B | —                               | "                       | 4              | 3 | 2 | 1 | —  |     |    |     |     |     | —                        |    |   |
| Gum arab. I | "      | 8-fold                          | "                       | 4              | 4 | 4 | 4 | 4  | 3   | 1  | —   |     |     | 2                        | —? | — |
| " " II      | "      | 8- "                            | "                       | 4              | 4 | 4 | 4 | 3  | 2   | —? | —   |     |     | (+)                      | —  |   |
| PVP         | "      | 8- "                            | "                       | 4              | 4 | 4 | 4 | 4  | 2   | 1  | —   |     |     | —                        |    |   |
| V           |        |                                 |                         |                |   |   |   |    |     |    |     |     |     |                          |    |   |
| —           | anti-A | —                               | "                       | 4              | 4 | 4 | 2 | 1  | —   |    |     |     |     |                          |    |   |
| —           | "      | —                               | anti-B; 4x <sup>3</sup> | 4              | 3 | 1 | — |    |     |    |     |     |     |                          |    |   |
| —           | anti-B | —                               | Saline                  | 4              | 4 | 3 | 1 | —  |     |    |     |     |     |                          |    |   |
| —           | "      | —                               | anti-A; 4x <sup>3</sup> | 3              | 2 | 1 | — |    |     |    |     |     |     |                          |    |   |

anti-A v. B cells; anti-B v. A<sub>1</sub> cells. <sup>2</sup> Strength of reaction. <sup>3</sup> Concentrated by dialysis against PVP.

The following »standard method» was worked out as a convenient way to obtain a five-fold concentration of serum<sup>1</sup> (see Fig. 2):

*Test Tubes.* — Small test tubes conventionally used in blood group work were cut to contain 0.5 ml.

*Dialysis Bag.* — A 30 cm long strip of dialyzer tubing was wetted and a knot was tied 6—8 cm from one end. A marble was dropped into the shorter end which was then closed by a knot. The 0.5 ml test tube was

<sup>1</sup> The arrangement was inspired by the »automatic» device for dialysis by Kohn (2). For routine work, concentrated solution of PVP was found to be more convenient than the dry powder used by Kohn.

TABLE 2  
CONCENTRATION OF SOME HUMAN RH-ANTISERA BY THE "STANDARD METHOD"

| Serum            | Test Cells               | Method <sup>1</sup> | Dilution of  |   |   |   |    |    |    |     |                    |    |   |   |    |    |    |     |
|------------------|--------------------------|---------------------|--------------|---|---|---|----|----|----|-----|--------------------|----|---|---|----|----|----|-----|
|                  |                          |                     | Native Serum |   |   |   |    |    |    |     | Concentrated Serum |    |   |   |    |    |    |     |
|                  |                          |                     | 1            | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 1                  | 2  | 4 | 8 | 16 | 32 | 64 | 128 |
| anti-D<br>(28)   | O, cDe/cde               | S                   | 4            | 4 | 3 | 1 | —  |    |    |     | 4                  | 4  | 4 | 3 | 3  | 1  | —  |     |
|                  | »                        | P                   | 4            | 4 | 4 | 4 | 2  | —? | —  |     | 4                  | 4  | 4 | 4 | 3  | 3  | —  |     |
|                  | O, cde/cde               | S, P                | —            |   |   |   |    |    |    |     | —                  |    |   |   |    |    |    |     |
|                  | A <sub>1</sub> , cde/cde | S                   | —            |   |   |   |    |    |    |     | —                  |    |   |   |    |    |    |     |
|                  | B, cde/cde               | S                   | —            |   |   |   |    |    |    |     | —                  |    |   |   |    |    |    |     |
| anti-D<br>(114)  | O, cDe/cde               | S                   | —            |   |   |   |    |    |    |     | —                  |    |   |   |    |    |    |     |
|                  | »                        | P                   | 3            | — |   |   |    |    |    |     | 4                  | 4  | 2 | — |    |    |    |     |
|                  | O, cde/cde               | S, P                | —            |   |   |   |    |    |    |     | —                  |    |   |   |    |    |    |     |
|                  | A <sub>1</sub> , cde/cde | S                   | —            |   |   |   |    |    |    |     | —                  |    |   |   |    |    |    |     |
|                  | B, cde/cde               | S                   | —            |   |   |   |    |    |    |     | —                  |    |   |   |    |    |    |     |
| Pooled<br>anti-D | O, cDe/cde               | S                   | —            |   |   |   |    |    |    |     | 4                  | 1  | — |   |    |    |    |     |
|                  | »                        | P                   | 4            | 4 | 4 | 4 | 3  | —? | —  |     | 4                  | 4  | 4 | 4 | 4  | 4  | 2  | —?  |
|                  | O, cde/cde               | S, P                | —            |   |   |   |    |    |    |     | —                  |    |   |   |    |    |    |     |
|                  | A <sub>1</sub> , cde/cde | S                   | —            |   |   |   |    |    |    |     | 3                  | —? | — |   |    |    |    |     |
|                  | B, cde/cde               | S                   | —            |   |   |   |    |    |    |     | 2                  | —  |   |   |    |    |    |     |

<sup>1</sup> S = saline

P = papainized cells

brought to the bottom of the long end of the strip and a rubber band was tied around the top of the tube to close the bag tightly around the tube opening.

*Procedure.* — 2.5 ml of serum was pipetted into the bag. With the help of a thin round-tipped glass rod air was removed from the small test tube to allow the serum fill the tube. The bag was closed with a knot, immersed in a cylinder filled with 40% PVP-solution (pH 7) and placed in cold room over night. The following day the bag was rinsed thoroughly with distilled water and cut open. The test tube contained 0.5 ml concentrated serum ready for use.

The above standard procedure was applied for study of a number of Rh-antisera. Typical experiments are given in Table II. An about four-fold specific increase in titer was usually obtained. Frequently (*e.g.* Table 2, pooled anti-D), concentration of human Rh-antisera revealed isoagglutinins which were present in native absorbed sera in concentrations below the detectable level.



## DISCUSSION

We are not aware of earlier work in which dialysis against viscous solutions would have been used to concentrate blood group agglutinins. Witebsky *et al.* (5) used dialysis against distilled water and obtained a protein precipitate, which mainly consisted of globulins. The separated and redissolved precipitate gave a higher titer of agglutinating Rh-antibodies than the original serum. In comparison to Witebsky's method, and others employing fractionation of proteins, the procedure described in this paper has the great advantage of simplicity. In addition, the undesirable precipitation of proteins is avoided. As pointed out above, the agglutination reactions were specific in up to 6-fold concentrated sera. When further concentration of agglutinins is desired, it is necessary to use fractionation procedures, the protein content of serum setting a limit to further concentration by our technique. We feel that the expansion of the titering range by this method could be utilized, *e.g.*: 1) To «improve» valuable but weak antisera. Undoubtedly, some low-titer sera, worthless as such, could be rendered useful through a 4—6 fold increase of the titer. 2) To check the specificity of weak antisera. For example, it certainly seems to be safe to use a low-titer serum in undiluted, native state if the serum is shown to be specific when checked in the concentrated state.

## SUMMARY

A 4 to 6-fold concentration of human anti-A<sub>1</sub>, anti-B, and anti-D (Rh<sub>0</sub>) sera by dialysis against a 40% solution of polyvinylpyrrolidone (Kollidon 17) resulted in a corresponding specific rise in the titers of the agglutinins. Dextran and gum arabic were also used for concentration. The possible applications of the technique were discussed.

*Acknowledgement.* — We are grateful for the technical assistance of Miss Kirsti Partanen.

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## ANGIOGRAPHY OF LABORATORY ANIMALS

by

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Angiography of laboratory animals is a valuable method in studying vascular anatomy and responses to experimental stimuli (1—5). Blood vessels can be visualized by injecting them with stained liquid latex or vinyl acetate, and by macerating the surrounding tissues with hydrochloric acid (10). Even if it is possible to study the functional state of the arteries with the aid of such a corrosion technique (7, 8), the radiographic angiographic technique is still superior owing to its simplicity, to the large focal depth and the high definition of the roentgen image, and the possibility to use stereoscopic technique (6). The present report deals with a new angiographic technique employing particulate contrast media.

### MATERIALS AND METHODS

The rat, the guinea pig and the rabbit were used as experimental animals. The animal to be examined was anesthetized with ether or with intravenously administered Urethane. A thin polythene catheter with an outer diameter of 0.6 mm was introduced via the aorta or the vena cava to the opening of the vessel to be filled with the radiopaque contrast agent. Only particulate contrast media were used which did not penetrate the capillary bed owing to their relatively large particle size. Most suitable of these agents were Chlorbismol (May and Baker) and Dionosil Aquaeous (Glaxo Laboratories, Ltd., Middlesex, England). The particle size

of both these media is below 20 micra, and arterioles and venules down to the size of about 40 micra were constantly filled.

After filling the vascular tree, the organ to be examined was removed for radiography. Frozen sections of about 1 mm were radiographed in some instances, but usually satisfactory results were obtained by radiographing the entire organ. A Siemens' »Fine Structure Roentgen Tube» equipped with a copper anode and a beryllium window was used. The focus-film distance was 30 cm, and the tube voltage used 20 to 50 kV. Crystallex fine-grained non-screen roentgen film or Kodaline Standard (Kodak) fine-grained film was used. The former allowed an enlargement by about 8 diameters, the latter about 30 diameters. The films were developed in Kodak's DX 80 X-ray developer or in D 76 fine-grain developer.

#### THORACIC AORTOGRAPHY

After laparotomy, the abdominal aorta was exposed and a thin polythene catheter inserted into it between two ligatures. The catheter was pushed upward, and the position of its tip was estimated by measuring the length of the introduced part. The contrast agent was injected slowly at a rate of about 0.5—1.0 cc per minute. In the beginning, the injection pressure was measured with the aid of a simple mercury manometer. After some training it was found possible to estimate the correct pressure. This was just high enough to press the contrast mass into the aorta through the thin catheter. The radiopaque mass entered the lumen of the aorta in droplets, and gradually filled the branch to be examined.

By injecting the contrast agent into the ascending aorta or the left cardiac ventricle, the coronary arteries could be filled. Injection into the arch of the aorta resulted in filling of the arteries of the neck and the head (Fig. 1) and the upper limbs. The intercostal arteries were filled by an injection into the descending aorta.

#### ABDOMINAL AORTOGRAPHY

The abdominal aorta was exposed and a catheter introduced into it. The position of the tip of the catheter was determined visually or by palpation, and the contrast agent was injected at



Fig. 1. — Arteriography of the neck and the head of the rabbit. 2 cc of Chlorbismol was injected through a polythene catheter slowly into the aortic arch. The carotid and the vertebral arteries are well visible as well as the cerebral arteries after the brains were removed and radiographed separately.

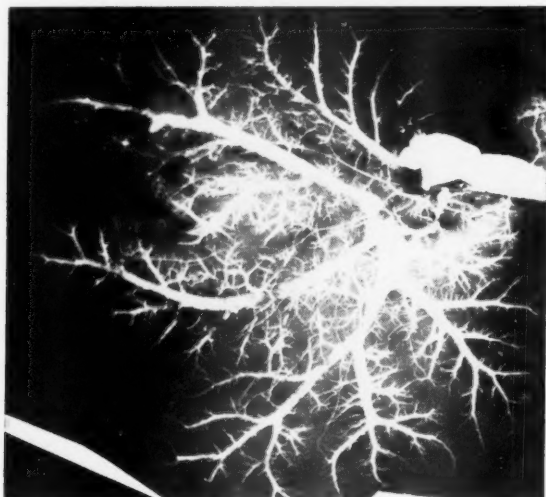


Fig. 2. — Hepatic venography of the rabbit. 2 cc of Chlorbismol was injected through a catheter inserted into the inferior vena cava at the opening of the hepatic vein. Slight overpressure was used, and the vana cava was compressed on both sides during the injection.

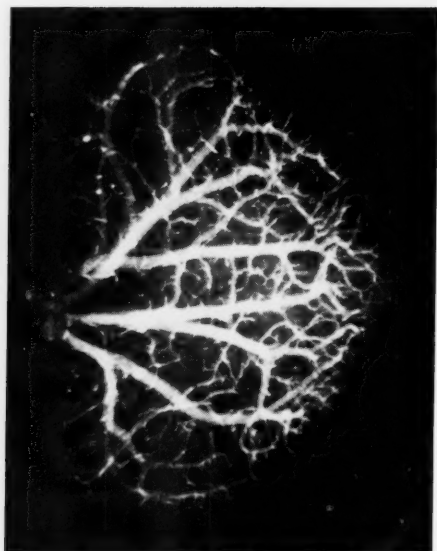


Fig. 3. — Renal arteriography of the rat, x 5. 1 cc of Dionosil Aqueous was injected at the level of the renal arteries through a catheter inserted into the abdominal aorta. The interlobar arteries are well visible, but the filling of the radiating cortical arteries is incomplete.

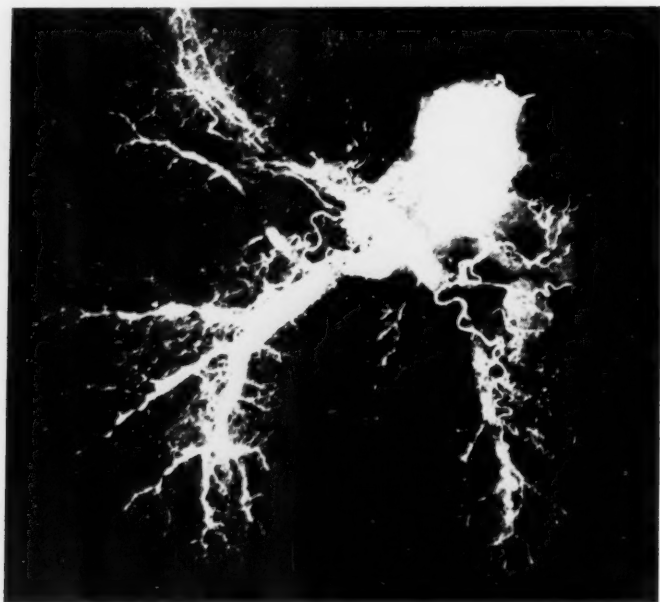


Fig. 4. — Pulmonary arteriography of the rat, x 4. 2 cc of Dionosil Aqueous was injected through a catheter introduced into the right atrium of the heart via the superior vena cava. There is a marked spasticity of the pulmonary arterial tree.

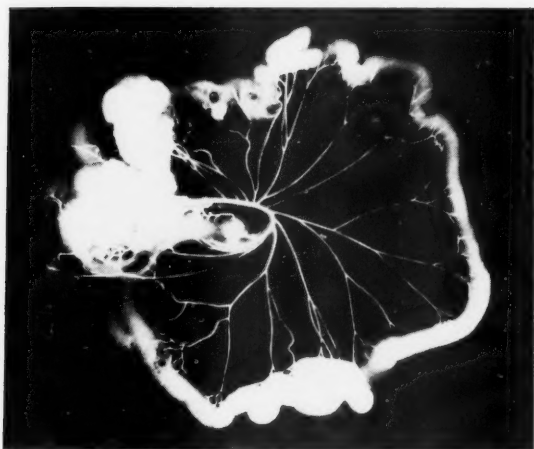


Fig. 5. — Mesenteric arteriography of the rat. 2 cc of Dionosil Aquaeous was injected at the opening of the mesenteric artery through a catheter inserted into the abdominal aorta. The arterial network of the intestinal walls is only partially filled.



Fig. 6. — Placentography of a guinea pig, x 3. 2 cc of Dionosil Aquaeous was injected at the origin of the uterine artery. The placental vessels are well visible, but the contrast agent does not pass to the fetal circulation.

the level of the artery to be examined. Thus, the branches of the coeliac axis were visualized, as well as those of the superior and the inferior mesenteric arteries (Fig. 5), the renal arteries (Fig. 3), and the arteries of the lower limbs. By filling the uterine artery of a pregnant female animal, the placental vessels could be visualized (Fig. 6).

#### CAVOGRAPHY AND PULMONARY ARTERIOGRAPHY

The superior vena cava was catheterized via the superficial jugular vein, which was exposed surgically. The tip of the catheter was pushed through the superior vena cava to the right atrium of the heart (11). The intracardiac position of the catheter tip was detected by its pulsation after having reached its aim. Injection of the contrast agent into the right atrium resulted in filling of the pulmonary arterial tree (Fig. 4). The injection was usually continued until the animal died. The lungs were radiographed in some instances after an injection of a small amount to the contrast agent. When the lungs were removed immediately after the injection, a part of the arterial tree was filled. After 2 to 3 hours the contrast agent had, however, disappeared.

The inferior vena cava was reached via the superior vena cava by pushing the catheter through the right atrium of the heart, or by exposing the inferior vena cava after laparotomy. The hepatic vein with its intrahepatic branches could be filled by injecting the contrast agent at its opening with a slight overpressure and by compressing the vena cava simultaneously on both sides (Fig. 2).

#### PORTOGRAPHY

The portal vein was exposed after laparotomy, and the contrast agent was injected into its wide distal end near the hepatic hilum through a thin needle. Attempts to insert a polythene catheter into this thin-walled vessel usually caused a profuse bleeding. The blood stream carried the contrast mass further, until it eventually filled the entire portal intrahepatic venous network.



## RESULTS AND DISCUSSION

The angiographic method described renders it possible to study arteries and veins down to the diameter of 40 micra. The vessels of this size are constantly filled with the particulate contrast media used, and a relatively simple radiographic method is needed for their demonstration. Because the particulate contrast media do not pass through the capillary bed, the arterial or venous network can be studied without harmful overlapping. The filling of the organ to be examined with the particulate contrast agent is extremely simple, because it automatically stops in small vessels, which makes the closure of the efferent vessels unnecessary. The microangiographic methods described by earlier authors (1, 9) still provide the best possibility for examination of the capillary structure, however, the vascular responses to external stimuli are often best demonstrable in relatively large blood vessels. The problem to be examined naturally determines the angiographic technique to be used.

As compared with the indirect methods, in which the blood flow and pressure are measured, the present method is rapid and simple and the direct visualization of the vascular responses is more dependable than their indirect measurements.

## SUMMARY

A simple and rapid angiographic method for examination of the vascular anatomy and responses of laboratory animals is described. Blood vessels down to the diameter of 40 micra are constantly filled with the particulate contrast media used, and a relatively simple radiographic technique is needed for their visualization. Thus the method is particularly suited for examination of small blood vessels, but does not provide any information on the capillaries.

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## EFFECT OF FLAVASPIDIC ACID AND MALE FERN EXTRACT ON GRAVIDITY

### II

AN EXPERIMENTAL STUDY OF MICE AND RABBITS, WITH  
SPECIAL CONSIDERATION OF THE DEVELOPMENT AND POSSIBLE  
MALFORMATION OF THE YOUNG

by

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(Received for publication March 30, 1961)

Flavaspidic acid and male fern extract are the drugs commonly used in Finland as vermifuges for broad tapeworm. Laboratory experiences indicate that these substances have some toxic effects on the circulatory system *in vitro* and *in vivo* (1, 2, 5, 6), and untoward effects also on the uterus and on the gravidity (3, 4). These substances administered *per os* were observed to have a highly abortive action. It was impossible, however, in these experiments to infer whether they have a disturbing effect on the development of the young in such cases when pregnancy does not end in abortion. In the following, we have tested the effect of flavaspidic acid and male fern extract administered during the early stage of pregnancy on the development of young mice and rabbits.

#### METHOD

The effect of flavaspidic acid and male fern extract on the development of young was tested on 61 white female mice and three white female laboratory rabbits.

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<sup>1</sup> Aided by a grant from the Mater Foundation.

The series of mice was divided into two groups (Table 1), the first comprising 37 mice and the second 24; the mice of the latter group were a so called second generation. Table 1 gives the doses of flavaspidic acid and male fern extract given by mouth to the mice in the two groups.

For mating, two male mice were put into a cage with ten female mice for ten days. Before mating, the weight of the female mice was 16—31 g. Weights were checked once weekly after the ten-days period, each female then being kept in a separate cage. Possible abortions were observed at the same time and abortus retentus verified at autopsy. Each female mice received the dose of drugs mentioned in Table 1 suspended in water through a gastric tube. The controls were given a corresponding quantity of pure water.

After administration of flavaspidic acid and male fern extract, the development of the embryo during pregnancy and possible malformations of the young caused by the drugs were studied as described in Table 1. During the whole period of study, the test animals obtained the diet usually given to laboratory animals.

The young of the first group were mated after the period of observation with the young in each group of drugs at the age of three and a half months (Table 1). This was done in order to find out whether any congenital malformations occurred in this generation of the young (the second generation).

On the fifth day after parturition, two of the rabbits (Table 2, Nos 1 and 2) were given 100 mg/kg of flavaspidic acid suspended in water through a stomach tube. The control animal received a corresponding quantity of water by the same means.

#### RESULTS

The results obtained in the study of the series of mice are given in Table 1. The corresponding results for the series of rabbits are included in Table 2.

On study of the results obtained in the series of mice of the first generation it is seen that when the mice were given flavaspidic acid, about half did not become pregnant, and in the extractum filicis series there were two definite cases of abortus retentus and two premature deliveries. There is a possibility, however, that, in spite of close observation, there may have occurred

TABLE 1  
MICE WERE USED AS TEST ANIMALS, DIRECTLY AFTER PARTURITION, THE ANIMALS WERE GIVEN A DOSE OF FILICIC DRUGS BY MOUTH AND THE CONTROLS A CORRESPONDING QUANTITY OF WATER

| I G E N E R A T I O N   |              |                         |                  |               |              |                  |      |                      |                        |                     |                 |                     |   |
|-------------------------|--------------|-------------------------|------------------|---------------|--------------|------------------|------|----------------------|------------------------|---------------------|-----------------|---------------------|---|
| Series                  | Total Number | Did not Become Pregnant | Abortus Retentus | Premat. Birth | Normal Birth | Young per Female |      | Young, average       |                        | Day of Opening Eyes | Surviving ♂ : ♀ | Congenital Malform. |   |
|                         |              |                         |                  |               |              | Born             | Died | Weight In g at Birth | Weight in g at 3 Weeks |                     |                 |                     |   |
|                         |              |                         |                  |               |              |                  |      |                      |                        |                     |                 |                     |   |
| Extr. filicis 0.5 mg/g  | 8            | 2                       | 2                | 3             | 1            | 6.0              | —    | —                    | 1.6                    | 6.0                 | 18.7            | 1 : 0.69            | — |
| Extr. filicis 0.25 mg/g | 5            | —                       | —                | —             | 5            | 4.8              | 1.8  | —                    | 1.2                    | 6.5                 | 17.8            | 1 : 1               | — |
| Flavasp. acid 0.1 mg/g  | 15           | 7                       | —                | —             | 8            | 6.1              | 0.8  | —                    | 1.5                    | 5.6                 | 16.9            | 1 : 0.84            | — |
| Water 1.5 ml            | 9            | 2                       | —                | —             | 7            | 6.9              | 1.3  | —                    | 1.3                    | 6.4                 | 17.1            | 1 : 1.11            | — |
| II G E N E R A T I O N  |              |                         |                  |               |              |                  |      |                      |                        |                     |                 |                     |   |
| Extr. filicis 0.5 mg/g  | 6            | 2                       | —                | —             | 4            | 5.0              | 0.5  | —                    | 1.3                    | 6.2                 | 17.8            | 1 : 0.80            | — |
| Extr. filicis 0.25 mg/g | 6            | 2                       | —                | —             | 4            | 5.0              | 0.3  | —                    | 1.5                    | 7.1                 | 18.4            | 1 : 1.11            | — |
| Flavasp. acid 0.1 mg/g  | 6            | 2                       | —                | —             | 4            | 6.3              | —    | —                    | 1.5                    | 6.3                 | 18.8            | 1 : 0.79            | — |
| Water 1.5 ml            | 6            | 1                       | —                | —             | 5            | 6.4              | 0.4  | —                    | 1.4                    | 6.9                 | 18.7            | 1 : 1.31            | — |

TABLE 2

RABBITS WERE USED AS TEST ANIMALS, FIVE DAYS AFTER PARTURITION THE ANIMALS WERE GIVEN FILICIC DRUGS BY MOUTH, AND THE CONTROL ANIMAL THE SAME QUANTITY OF WATER

| Series                     | No. of Rabbit | Normal Birth | Young |      | Average Weight at 15 Days in g | Weight at 50 Days in g | Surviving ♂ : ♀ | Congenital Malformat. |
|----------------------------|---------------|--------------|-------|------|--------------------------------|------------------------|-----------------|-----------------------|
|                            |               |              | Born  | Died |                                |                        |                 |                       |
| Flavaspidic acid 100 mg/kg | 1             | +            | 3     | —    | 290                            | 1450, 1420, 1400       | 2 : 1           | —                     |
| Flavaspidic acid 100 mg/kg | 2             | +            | 5     | 2    | 160                            | 1240, 1240, 1340       | 2 : 1           | —                     |
| Control                    | 3             | +            | 6     | 4    | 170                            | 880, 1220              | 2 : 0           | —                     |

early abortion of a fertile egg which has escaped macroscopical notice. These observations agree with those made in our previous investigations in that male fern extracts have a highly abortive effect. The number of young born to each female or those who died in each series cannot be distinctly distinguished when there is a question of mature delivery. No difference in increase in weight or time of opening the eyes in the mature young was noted as compared with controls. On close study of the results of this investigation, it seems evident that the drugs cannot have caused congenital malformations although comparatively large doses were given at an early stage of pregnancy. In the groups of drugs, there were in general less female young than males, whereas in the controls, the occurrence of the two sexes was the opposite. The frequency of males and females was similar in the series of the second generation of mice. In other respects, the development of the young of the second generation was comparable with that in the controls.

The series of rabbits was small, hence the observations made do not suffice for reliable conclusions.

#### DISCUSSION

In our previous investigations it has been noted that male fern extract and flavaspidic acid have a comparatively strong disturbing effect on pregnancy in that abortion (*abortus retentus*) is caused, and in the fetus born alive, the young have been underweight and weak. On the account there was reason to continue investigations most closely for observation of the surviving young of the test animals and to study whether the vermifuge in question may cause congenital malformations or other developmental disturbances. There is a possibility that in some parts of Finland, vermifuges are taken also during pregnancy and there may thus be a danger of and injurious effect on the development of the fetus. Evaluation of the results obtained in the present tests seem, however, to reveal that the male fern extracts as such do not affect the fetus in any considerable degree in the cases when abortion or death of the fetus does not occur. Naturally the results in these tests on animals cannot, however, be directly applied to clinical medicine.

## SUMMARY

The effect of peroral administration of flavaspidic acid and male fern extract to test animals were studied with regard to the development of their young and especially to congenital malformations. A tendency to disturbances during pregnancy was observed, like in our previous investigations, but no post partum malformations were noted in young.

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## FACTORS AFFECTING THE BACTERIAL COUNT ON THE TEETH SURFACES

by

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If a tooth loses its neighbour in the middle of an arch, its caries frequency decreases.

In this work the effects of the loss of a neighbour on the bacterial count of the teeth surfaces were investigated.

### MATERIAL AND METHODS

The lactobacilli were counted on the buccal surface of a tooth without a neighbour as well as of the corresponding tooth on the other side in the undamaged arch. 15 patients from the Institute of Dentistry were examined.

The technique for counting lactobacilli was previously described. (1)

### RESULTS

In most cases (13/15) the lactobacillus count of the buccal surface on the tooth without an adjacent one was lower than that of the corresponding tooth on the other side (Table). In two cases, however, the results were the opposite.

### DISCUSSION

According to our results it is quite possible that the gap in the arch could have a cleaning effect on the buccal surface of the



TABLE  
RELATIVE LACTOBACILLUS COUNT OF THE BUCCAL SURFACES OF THE TEETH  
EXAMINED

| Number of Case | Tooth Next to a Gap | Control | The Status of Caries and Fillings of |                  | Relative Lacto-bacillus Count of the Buccal Surface of |         |
|----------------|---------------------|---------|--------------------------------------|------------------|--|---------|
|                |                     |         | Tooth Next to a Gap                  | Control          | Tooth Next to a Gap                                    | Control |
| 1              | 5—                  | —5      | —                                    | —                | 1080   | 1913    |
| 2              | —5                  | 5—      | —                                    | —                | 635  | 3167    |
| 3              | 4—                  | —4      | —                                    | —                | 110  | 2753    |
| 4              | +4                  | 4+      | a <sup>14</sup>                      | a <sup>124</sup> | 1920   | 11867   |
| 5              | 3—                  | —3      | —                                    | —                | 0  | 85      |
| 6              | 4—                  | —4      | —                                    | —                | 0  | 100     |
| 7              | +5                  | 5+      | —                                    | —                | 465  | 2290    |
| 8              | 6+                  | +6      | —                                    | —                | 0  | 0       |
| 9              | +4                  | 4+      | —                                    | —                | 285  | 510     |
| 10             | 4+                  | +4      | —                                    | a <sup>14</sup>  | 487  | 95      |
| 11             | —5                  | 5—      | c <sup>9</sup>                       | —                | 13323  | 5370    |
| 12             | 6+                  | +6      | —                                    | —                | 5830   | 7350    |
| 13             | 4—                  | —4      | c <sup>4</sup>                       | c <sup>4</sup>   | 3030   | 6403    |
| 14             | —5                  | 5—      | a <sup>14</sup>                      | a <sup>14</sup>  | 1310   | 1543    |
| 15             | +4                  | 4+      | a <sup>14</sup>                      | a <sup>124</sup> | 195  | 385     |

adjacent tooth. This cleaning of the buccal surface might result from the easy flow of saliva and food debris through the gap in the arch. In one of the exceptional cases the tooth in question had cervical caries. For the other exception we have no explanation.

#### SUMMARY

The effects of the missing of the tooth on the bacterial count of the adjacent tooth were studied. The lactobacillus count was in general high if the arch was undamaged and low if an adjacent tooth was missing.

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## OVARIAN HILUS CELLS AND UTERINE PATHOLOGY

by

HANNES SAURAMO

(Received for publication March 28, 1961)

In a previous study (2) of the histology and function of the ovaries in genital pathological and general pathological cases, attention was directed also the occurrence of ovarian hilus cells. It was found that the presence of ovarian hilus cells seems to be fairly typical in cases of uterine myoma. The present work was undertaken with the object of studying hilus cells in cases of uterine pathology in general, and to check their occurrence in cases of uterine myoma.

### MATERIAL AND METHODS

The series consisted of 50 cases. The ovarian hilus was examined bilaterally on the basis of serial sections or incomplete serial sections. In addition, a few sections were prepared from other parts of the ovaries for a better overall picture. The fixing agent was 10 per cent formol. All material was freshly obtained. The staining methods were Weigert's Iron Haematoxylin-van Gieson stain, and Ehrlich Haematoxylin-Nuclear Fast Red. The term »second ovary» refers to the ovary in which hilus cells were generally present to a less extent.

### RESULTS

*Uterine Myoma.* — Thirty cases were studied. Ages varied from 40 to 55 years, with the exception of one subject aged 68. Hilus cells occurred as follows.

There were 10 cases in which ovarian hilus cells were seen in large numbers, or were large or distinctly visible; in 2 of these cases crystalloids were noted. The second ovary showed hilus

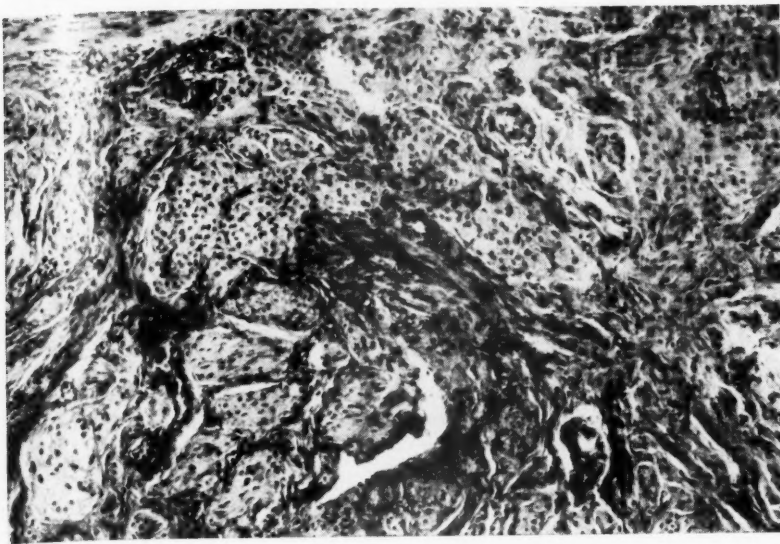


Fig. 1. — 43 years. Uterine myoma. Ovarian hilus cells. +100.

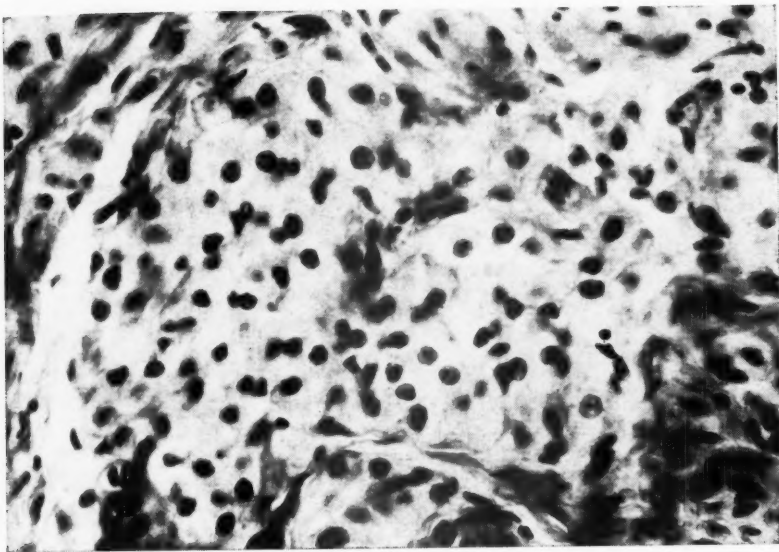


Fig. 2. — 43 years. Uterine myoma. Ovarian hilus cells. + 400.

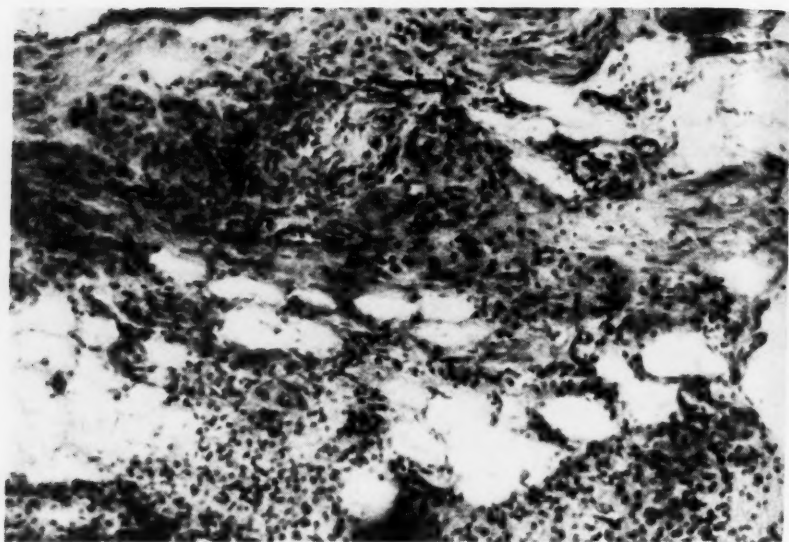


Fig. 3. — 51 years. Uterine myoma. Ovarian hilus cells. + 200.

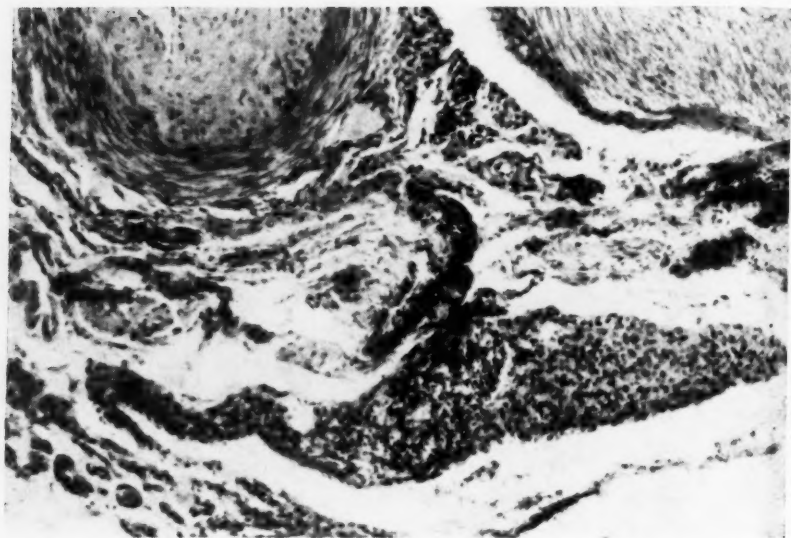


Fig. 4. — 47 years. Uterine myoma. Ovarian hilus cells. + 100.

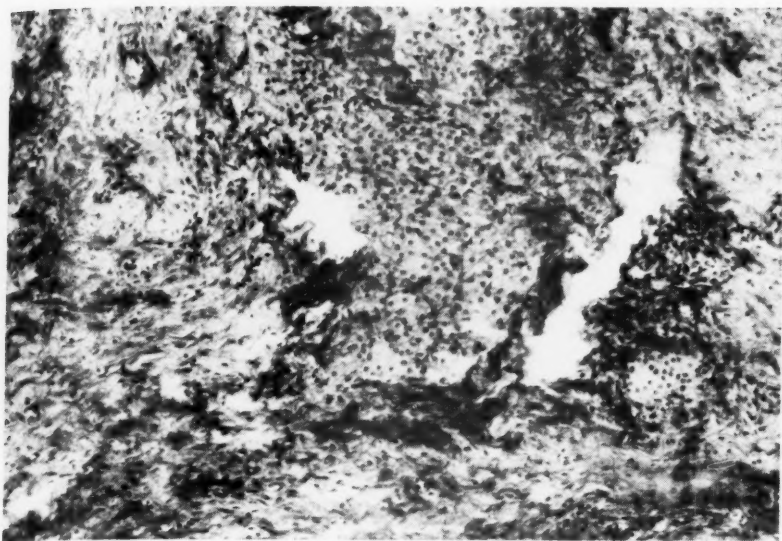


Fig. 5. — 61 years. Endometrial cancer. Ovarian hilus cells.  $\times 100$ .

cells distinctly or at least to some extent in 7 cases, and no hilus cells in 3.

There were hilus cells which were of atrophic appearance or incompletely developed in 17 cases. For the second ovary this applies to 9 cases, while in 8 cases hilus cells were absent.

Hilus cells were bilaterally absent in 3 cases.

The connective tissue was usually dense in the ovarian cortex. In the inner portions dense connective tissue islands occurred to a variable extent.

There was no correlation between age, occurrence of hilus cells and connective tissue islands. However, some kind of relationship was observed between the right and left ovary in regard to occurrence of hilus cells.

*Uterine Hyperplasia or Cystic Glandular Hyperplasia of the Endometrium.* — The number of cases studied was 6. Ages varied from 44 to 53 years. Hilus cells were distinctly present in 4 cases, one of which showed crystalloids. Hilus cells of atrophic appearance were seen in 2 cases. In other respects the results were in line with those reported for uterine myoma.

*Uterine Sarcoma.* — Two cases were studied. Hilus cells were

present in small number in the ovaries of a 37-year-old patient, and distinctly in those of a 50-year-old patient.

*Carcinoma of Endometrium.* — The cases numbered 11. The patients varied in age from 31 to 66 years.

Hilus cells occurred in large numbers or distinctly in 6 cases. The second ovary showed hilus cells distinctly or to a slight extent in 2 cases, and no hilus cells in 4 cases.

Atrophic-looking hilus cells were present in 2 cases. In one of these, there were also atrophic hilus cells in the second ovary.

Hilus cells were absent bilaterally in 3 cases. In other respects the results were similar to those reported for uterine myoma. The ovaries contained carcinomatous tissue in 2 cases.

*Carcinoma of Cervix.* — In one grade 1 case there were no hilus cells in the ovaries.

#### DISCUSSION

My previous studies dealing with the occurrence of hilus cells (1, 2) included also reviews of the literature. The earlier and recent literature will not be dealt with here in greater detail seeing that further studies on ovarian hilus cells are in progress and that I will have to revise my earlier conclusions in certain respects.

An observation made by Sherman and Woolf (3) should be mentioned, however. They found that hilus cell hyperplasia was present in 80–100 per cent of the cases of endometrial carcinoma studied. They believe in a hormonal origin of endometrial cancer.

As stated above, ovarian hilus cells are common in cases of uterine pathology. The accompanying figures illustrate these observations.

#### SUMMARY

A study of 50 cases revealed that ovarian hilus cells were present more or less distinctly in the majority of cases of uterine myoma, uterine and endometrial hyperplasia, and endometrial cancer. This evidently also applies to uterine sarcoma.

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## OVARIAN HILUS CELLS AND OVARIAN PATHOLOGY

by

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It was concluded in my earlier report on ovarian hilus cells (4) that additional studies are required regarding the correlation between cystadenoma and hilus cells. It is the purpose of the present work to throw more light on this question, dealing at the same time with the occurrence of hilus cells in ovarian pathology in general.

### MATERIAL AND METHODS

The study was based on 40 cases. The ovarian hilus was examined unilaterally or, if possible, bilaterally. Complete and incomplete serial sections were prepared. In addition, a few sections were prepared from other parts of the ovaries for a better overall picture. As regards fixing and staining a description will be found in my report on ovarian hilus cells and uterine pathology (5). The second ovary refers to the ovary in which there were generally less hilus cells.

### RESULTS

*Ovarian Fibroma, Adenofibroma, Fibromyoma, Thecomatosis and Thecoma.* — Seven cases were studied. The ages of the patients varied from 32 to 69 years. Ovarian hilus cells were present in large numbers or distinctly in 4 cases. In one case (thecoma) there were crystalloids. Hilus cells were absent in 3 cases.



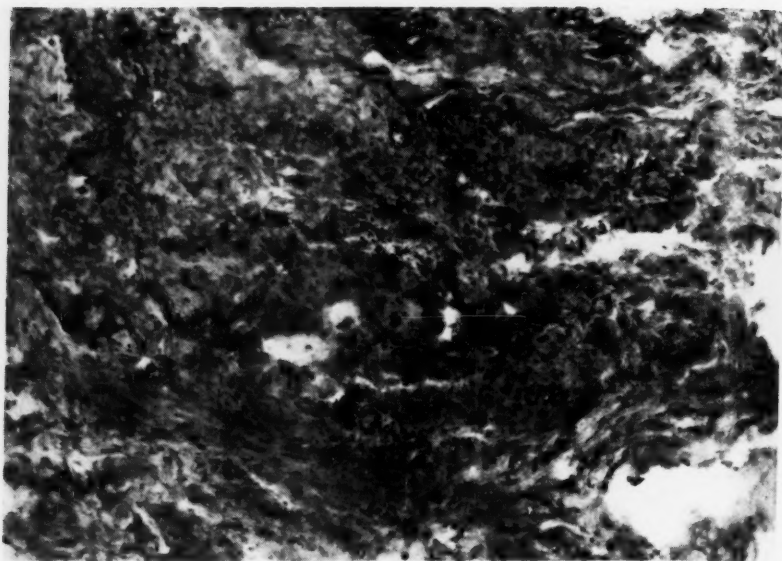


Fig. 1. — 47 years. Ovarian mucinous cystadenoma. Ovarian hilus cells.  $\times 100$ .

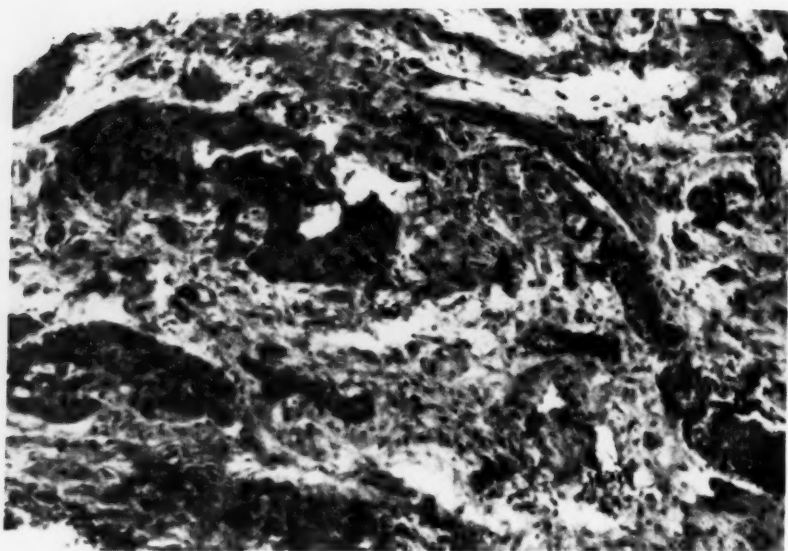


Fig. 2. — The same case,  $\times 100$ .



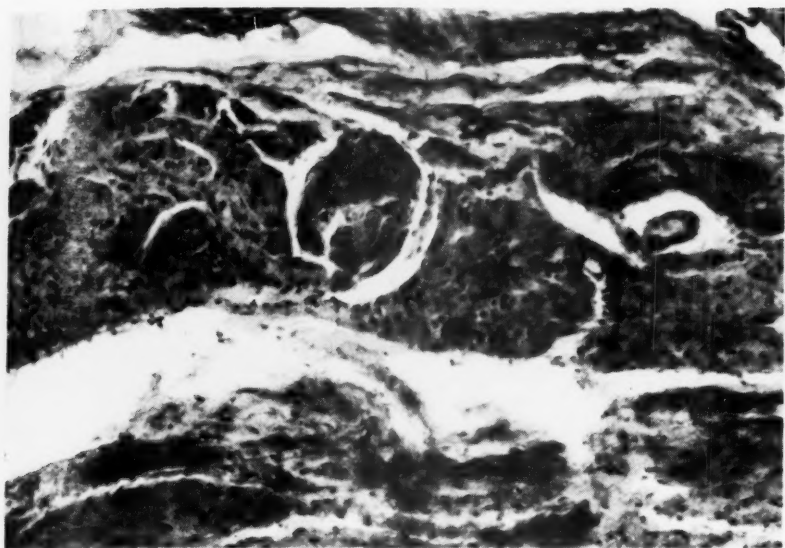


Fig. 3. — 54 years. Ovarian thecoma. Ovarian hilus cells. + 100.



Fig. 4. — 54 years. Breast cancer. Ovarian hilus cells. + 100.

Connective tissue islands were probably most distinct in the cases associated with hilus cells. There seemed to be no correlation between age and the presence of hilus cells — apart from the fact that these cells were not found in persons under 40 years. The second ovary was available for examination in 3 cases. Hilus cells seemed to occur bilaterally.

*Dermoid Cysts.* — Hilus cells were not demonstrated in the 2 cases studied (25 and 31 years). The second ovary was not available for examination.

*Unilocular Ovarian Cysts.* — Four cases were studied; the ages of the patients varied from 31 to 67 years. Hilus cells were present to some extent in 2 cases, and in 2 cases these cells did not occur. The second ovary could be examined in one case, which showed a small number of hilus cells.

*Serous Ovarian Cystadenoma.* — The 8 subjects examined varied in age from 29—65 years. Hilus cells were distinctly present in 4 cases, they were of atrophic appearance in 2 cases, and absent in 2 cases. In these 8 cases no hilus cells appeared in persons under 40 years. The second ovary was not available for examination.

*Ovarian Mucinous Cystadenoma.* — The 8 cases studied included patients from 39 to 56 years old. Hilus cells occurred distinctly in 4 cases; two of these showed crystalloids. Hilus cells were present to some extent in 2 cases, and there were atrophic-looking hilus cells in one additional case. No hilus cells were demonstrated in one case, and this was the single case in which also the second ovary could be examined. The youngest subjects showed only few hilus cells.

*Ovarian Cancer.* — The 4 subjects examined varied in age from 47 to 50 years. Distinct hilus cells were seen in 2 cases, and these cells were absent in 2 cases. The second ovary was available for examination in 3 cases, and it showed no hilus cells. Cancer tissue was found in all the 3 cases studied.

*Ovarian Granulosa-Cell Cancer.* — In the 2 cases examined, which showed sarcomatous features, the subjects varied in age from 62 to 66 years. Hilus cells were not found. In one case the second ovary could be examined; this case showed no hilus cells but did show cancer tissue.

*Malignant Ovarian Teratoma.* — In the one case examined, a patient aged 41, there appeared no hilus cells. The second ovary was not available.

*Hyperoestrogenism.* — Four cases were studied; the subjects varied from 53 to 54 years. All cases were diagnosed by hormone assay and there was no tumour. Hilus cells were distinctly present in 3 cases and absent in one. The second ovary showed some hilus cells in 2 cases and none in 2. Connective tissue was of large amount in the ovaries. In 2 cases there were cysts of the follicle system.

*Breast Cancer.* — Five cases of breast cancer were also studied. Ages varied from 34 to 56 years. The ovaries contained hilus cells in large number or distinctly in 2 cases, in one of these bilaterally. The hilus cells were atrophic in appearance or present in small number in 2 cases (in one bilaterally). One also bilaterally examined case showed no hilus cells. The patient was under 40 years. One of the cases was a Krukenberg tumour, and hilus cells were present in small number in one ovary. In none of the other cases was cancer tissue present in the ovaries.

#### DISCUSSION

More detailed studies of the occurrence of hilus cells in cases of ovarian pathology have not been performed. Some case reports should be referred to. Hilus-cell and granulosa-cell tumours in the same ovary (1) have been described; female sex chromatin was present in the nuclei of the hilus cells. A case of interstitial cell hyperplasia of the ovary was associated with a Brenner tumour (2), and there was no definite evidence of masculinization. Published reports further include one granulosa cell tumour containing Leydig cells (3).

It was stated above that hilus cells are common in connection with ovarian tumours. They do not, however, seem to be frequent in association with the following tumours: ovarian dermoid cysts, unilocular cysts, granulosa cell tumour, and malignant teratoma. Hilus cells were often noted in cases of breast cancer. The accompanying figures illustrate the findings reported.

#### SUMMARY

In 40 cases studied, ovarian hilus cells occurred more or less distinctly in the majority of subjects with ovarian serous cystadenoma and ovarian mucinous cystadenoma. Hilus cells were

common in connection with tumours of ovarian fibroma type, especially in the presence of thecal cell hyperplasia. The same applies to ovarian cancer, hyperoestrogenism and cysts of the follicle system. Cases of breast cancer were often associated with ovarian hilus cells. The presence of hilus cells does not seem to be typical in ovarian dermoid cysts.

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## BACTERICIDAL EFFECT OF SERUM FROM NORMAL CHICKENS AND CHICKENS WITH ROUS SARCOMA

by

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The bactericidal effect of fresh serum has long been recognised (1, 9). In 1954, Pillemer and his co-workers advanced the properdin theory according to which the non-specific bactericidal effect of serum depends on the action of the 4 factors of the complement and a new protein, properdin, in the presence of magnesium (11). According to Pillemer and his co-workers (6), it was possible to induce increased resistance to bacterial infections in mice *in vivo* through lipopolysaccharide injections and to demonstrate concurrently an elevated properdin titre. Pillemer concluded that the increased resistance was caused by an increase in the properdin content.

The possibility that a lowered properdin content might be associated with reduced resistance to intercurrent infections in various diseases such as cancer, leukemia, lymphogranulomatosis, etc. prompted several studies of the properdin content in the sera of patients with various diseases and of normal men and experimental animals of different ages (3, 4, 5, 7, 13, 14).

It has been shown for instance (3) that patients with cancer have almost without exception an abnormally low properdin content and that the further the disease has progressed the lower is the properdin content.

The properdin content in albino rats with 3.4 benzpyrene sarcoma was studied by Modica and Fumarola (7) who established that the further the tumour had advanced the lower was the properdin content, and that finally it was immeasurable.

In a study of normal sera *in vitro* Pillemer (15) pointed out that the bactericidal effect of serum against gram-negative bacteria was dependent on the properdin system. But the connection between similar changes in the properdin level *in vivo* in cancer and other diseases and the *in vitro* bactericidal effect of the serum is nevertheless not clear.

The *in vitro* bactericidal effect of human serum in different diseases in which a low properdin content has been demonstrated, *e.g.* cancer, leukemia, lymphogranulomatosis, was consequently studied by Roantree and Rantz (12). Among others they could demonstrate no difference between sera from these patients and healthy subjects.

This suggested the need for an investigation of the bactericidal effect of serum from experimental animals bearing an experimentally induced tumour. The present paper is a report on the results of an investigation of the bactericidal effect of serum from chickens of different ages with far-advanced Rous sarcoma compared with the bactericidal effect of serum from normal chickens. Sera from starved chickens were studied as controls primarily to establish the possible effect of stress.

#### MATERIAL AND METHODS

*Chickens.* — White Leghorn chickens from the same poultry yard were used throughout the experiment.

*Tumour.* — The Rous sarcoma virus used was originally obtained from Dr. R. J. C. Harris, Imperial Cancer Research Fund, England.

Ten per cent suspensions in amounts of 0.1 ml were injected into the thigh of day-old chickens and a new virus suspension was prepared according to the method of Carr and Harris (2) of the tumours that originated.

This suspension was diluted to 10 per cent and injected into the thigh of chickens in groups a and b (0.1 and 0.2 ml, respectively) and into the pectoral muscle in groups c and d (0.5 and 1.0 ml, respectively).

The chickens became cachectic 12–18 days after the inoculation. The blood was drawn as late as possible. In this way, because of the individual variations in the rate of growth of the tumour, all the chickens of a given group did not die on the same day.

The starved chickens were given no food for c. 3 days, but were allowed water ad libitum. Their general condition consequently corresponded roughly to that of the Rous group.

*Blood Samples.* — The blood was taken sterilely by heart puncture. The sample was kept for 2 hours at room temperature and then overnight at  $+4^{\circ}\text{C}$ , after which it was centrifuged. The serum was poured into sterile Kahn tubes which were closed with rubber stoppers and deep-frozen at  $-60^{\circ}\text{C}$ .

*Bacterial Strain.* — *Shigella Dysenteriae* ATCC 9665 which has previously been used for corresponding experiments (15) was employed in the present work. The strain was kept on ordinary agar at  $+2^{\circ}\text{C}$  and subcultures were made once a month.

*Buffer.* — The buffer used was Dulbecco's phosphate buffer (Orion) which is available commercially and in which the number of bacteria/ml of buffer remained unchanged after incubation for 90 min. in a water bath at  $37^{\circ}\text{C}$ .

*Method of Testing.* — From a colony on agar a culture was made in broth. This was for practical reasons incubated at  $37^{\circ}\text{C}$  for 23 hours. This procedure gave a fairly constant bacterial concentration. The broth suspension actually used in the test was prepared daily. Ordinary agar plates were used as culture media for counting the colonies.

A two-fold dilution series was made of the serum with buffer 1/1—1/32. Into each tube was introduced 250 bacteria ( $\pm 20$  per cent), whereupon the whole series was incubated in a water bath at  $37^{\circ}\text{C}$  for 90 min. A pilot experiment had shown that it was of no account if 250 or more bacteria (up to 2,500) were used in the test conditions.

The counting of bacteria was made by spreading 0.1 ml from each serum dilution tube on an agar plate. The agar plates were incubated overnight at  $37^{\circ}$  and the colonies counted. Three controls were made with buffer instead of serum in each experiment. Samples were taken from the controls before and after incubation and the bacterial count remained constant.

If no inhibition occurred in the presence of the serum a c. three-fold increase in the amount of bacteria was obtained after 90 min. This is denoted in the tables by — (= no bactericidal or bacteriostatic effect). In certain cases the number of bacteria in the presence of serum agreed with that in the controls in which buffer alone was



used. This is denoted by  $\pm$  (= bacteriostatic effect), and when the number of colonies was less than 1/3 of that of the controls the result is denoted by + (= bactericidal effect). The difference between + and — is thus at least nine-fold.

## RESULTS

*Bactericidal Effect of Serum from Normal, Tumour-bearing and Fasting Chickens.* — Each experimental group consisted of 6–12 chickens, the controls of the same number and the fasting groups of about 6 chickens. It was necessary for practical reasons to pool 2–3 sera in the youngest age groups.

Rous sarcoma virus was inoculated into chickens of (a) 1 day, (b) 2 weeks, (c) 4 weeks and (d) 10 weeks of age. The chickens

TABLE 1

THE BACTERICIDAL EFFECT OF SERUM FROM 5 WEEKS OLD CHICKENS A) BEARING ROUS SARCOMA B) NORMAL C) AFTER 3 DAYS OF STARVATION

|                            | No. | Serum Dilution |       |       |       |       |      |
|----------------------------|-----|----------------|-------|-------|-------|-------|------|
|                            |     | 1/1            | 1/2   | 1/4   | 1/8   | 1/16  | 1/32 |
| Chickens with Rous sarcoma | 1   | +              | +     | +     | +     | $\pm$ | —    |
|                            | 2   | +              | +     | $\pm$ | —     | —     | —    |
|                            | 3   | +              | +     | —     | —     | —     | —    |
|                            | 4   | +              | —     | —     | —     | —     | —    |
|                            | 5   | $\pm$          | —     | —     | —     | —     | —    |
|                            | 6   | —              | —     | —     | —     | —     | —    |
| Normal chickens            | C1  | +              | +     | +     | +     | +     | —    |
|                            | C2  | +              | +     | +     | +     | $\pm$ | —    |
|                            | C3  | +              | +     | +     | +     | $\pm$ | —    |
|                            | C4  | +              | +     | +     | $\pm$ | —     | —    |
|                            | C5  | +              | +     | $\pm$ | —     | —     | —    |
|                            | C6  | +              | $\pm$ | —     | —     | —     | —    |
|                            | C7  | —              | —     | —     | —     | —     | —    |
| Starved chickens           | S1  | +              | +     | +     | —     | —     | —    |
|                            | S2  | +              | +     | —     | —     | —     | —    |
|                            | S3  | +              | —     | —     | —     | —     | —    |
|                            | S4  | +              | —     | —     | —     | —     | —    |
|                            | S5  | +              | —     | —     | —     | —     | —    |
|                            | S6  | —              | —     | —     | —     | —     | —    |

+ = Bactericidal effect  
 $\pm$  = Bacteriostatic effect  
 — = No effect



became cachectic after 12—18 days. The blood samples were taken at (a) 2 weeks, (b) 5 weeks, (c) 7 weeks and (d) 12 weeks. Food was generally withheld from the fasting groups for 3 days before sampling.

Great individual variations were noted in the different groups (Table 1). An exception was the group which had been inoculated at the age of 10 weeks (samples taken at 12 weeks) and its controls. A high bactericidal effect was established throughout this age group (Table 2).

TABLE 2

THE BACTERICIDAL EFFECT OF SERUM FROM 12 WEEKS OLD CHICKENS A) BEARING ROUS SARCOMA B) NORMAL C) AFTER 3 DAYS OF STARVATION

|                            | No. | Serum Dilution |     |     |     |      |      |
|----------------------------|-----|----------------|-----|-----|-----|------|------|
|                            |     | 1/1            | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 |
| Chickens with Rous sarcoma | 1   | +              | +   | +   | +   | +    | +    |
|                            | 2   | +              | +   | +   | +   | +    | ±    |
|                            | 3   | +              | +   | +   | +   | +    | ±    |
|                            | 4   | +              | +   | +   | +   | +    | ±    |
|                            | 5   | +              | +   | +   | +   | ±    | —    |
|                            | 6   | +              | +   | +   | ±   | ±    | ±    |
|                            | 7   | +              | +   | +   | ±   | ±    | —    |
|                            | 8   | +              | +   | +   | ±   | ±    | —    |
| Normal chickens            | C1  | +              | +   | +   | +   | +    | +    |
|                            | C2  | +              | +   | +   | +   | +    | ±    |
|                            | C3  | +              | +   | +   | +   | +    | —    |
|                            | C4  | +              | +   | +   | +   | +    | —    |
|                            | C5  | +              | +   | +   | +   | ±    | ±    |
|                            | C6  | +              | +   | +   | +   | ±    | ±    |
|                            | C7  | +              | +   | +   | +   | ±    | —    |
|                            | C8  | +              | +   | +   | +   | ±    | —    |
|                            | C9  | +              | +   | +   | +   | ±    | —    |
| Starved chickens           | S1  | +              | +   | +   | +   | +    | +    |
|                            | S2  | +              | +   | +   | +   | +    | ±    |
|                            | S3  | +              | +   | +   | +   | +    | ±    |
|                            | S4  | +              | +   | +   | +   | +    | ±    |
|                            | S5  | +              | +   | +   | +   | +    | ±    |
|                            | S6  | +              | +   | +   | +   | +    | ±    |

+= Bactericidal effect

± = Bacteriostatic effect

— = No effect

Although the normal group in Table 1 showed a slight tendency to higher values, no significant difference was demonstrated in any age group between normal chickens, Rous chickens and starved chickens.

*Bactericidal Effect of Serum from Normal Chickens of Different Ages.* — Comparison of the bactericidal effect of serum from normal chickens of different ages showed great individual variations in all groups up to 12 weeks. However, a high bactericidal titre was demonstrated in the last-mentioned group as a whole (Table 2 and Fig. 1).

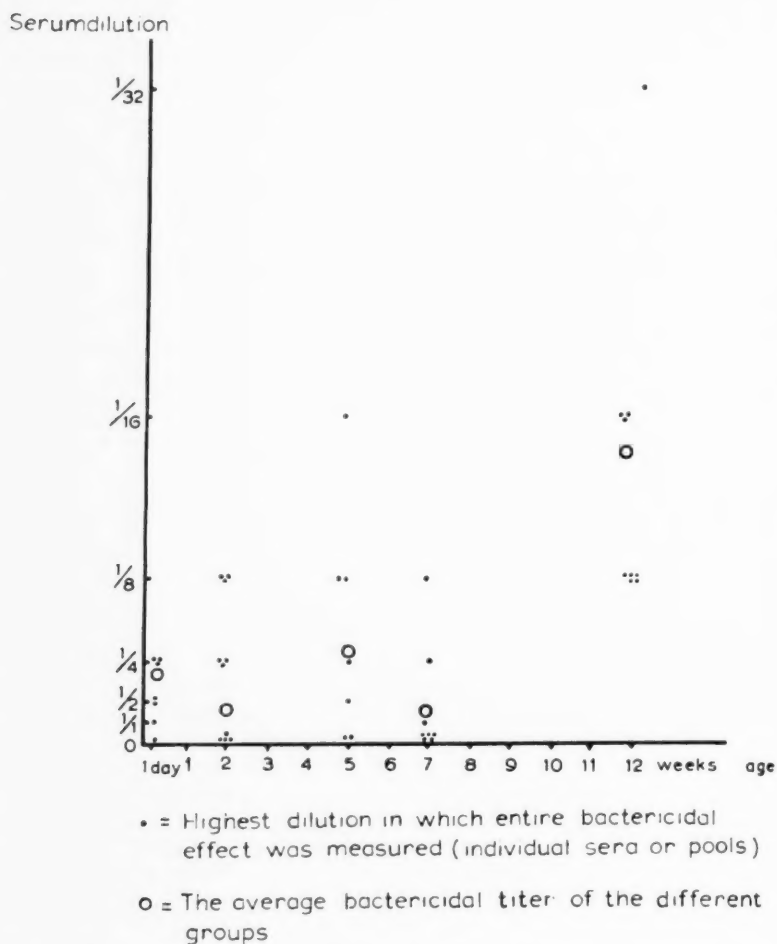


Fig 1. Bactericidal effect of serum from normal chickens of different ages.

As can be seen from Fig. 1, the bactericidal effect varied in the younger groups from no effect in undiluted serum to full bactericidal effect in 1/8 dilution (in one case 1/16). By contrast, all sera in the 12 weeks group had full bactericidal effect in 1/8 dilution and one serum even in 1/32 dilution.

Although the material is small this observation indicates that the bactericidal effect of chicken serum increases with age. The small variations in the mean values seen in the younger age groups (Fig. 1) cannot apparently be regarded as significant.

#### DISCUSSION

The fact that it was not possible to establish a significant difference in bactericidal effect in serum from normal chickens on the one hand and chickens infected with Rous sarcoma virus on the other hand supports, in so far as parallels can be drawn between sera from so different species, the observation made by Roantree and Rantz who were not able to demonstrate any such difference between human sera from patients with cancer and other serious diseases and normal human sera.

By contrast, there are reports on a sharp fall in the properdin content of patients and experimental animals with cancer in the terminal stage (3, 7, 13, 14).

If a similar decrease in the properdin level of chickens with Rous sarcoma could be expected, the finding that the Rous sarcoma did not influence the bactericidal effect of serum in these experiments seems to suggest that there is no correlation between possible decreases in the properdin content and the bactericidal effect measured with *Shigella Dysenteriae*. Reliable conclusions on this point would, of course, require parallel properdin determinations.

It has been suggested that specific antibodies play a role in the genesis of the bactericidal effect of serum against gram-negative bacteria (8, 10, 16). The increasing bactericidal effect which was established in the present work at a higher age could, if this is the case, depend on a gradual increase of these antibodies. The great individual variation in the younger groups might possibly be attributed to the production of antibodies at different rates or be thought

to reflect a variation in the rate of maturation of the system which produces the proteins necessary for the bactericidal effect of the serum.

#### SUMMARY

No difference was established in the bactericidal effect against *Shigella Dysenteriae* between serum from normal chickens, chickens with Rous sarcoma and starved chickens.

A great individual variation occurred in the groups of normal chickens up to 7 weeks of age. A consistent high bactericidal effect was established in chickens aged 12 weeks.

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## THE COMBINED EFFECT OF CERTAIN SURFACE-ACTIVE AGENTS AND ANTIBIOTICS

by

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It is well known that the resistance which bacteria develop to the antibiotics has to a great extent interfered with the treatment of infections. However, it is frequently possible by the addition of certain agents to enhance the potency of antibiotics. The present writer has studied the effect of certain surface-active agents on the ability of the most common antibiotics to inhibit the growth of staphylococci on agar, and the results are presented here.

Even before the antibiotic era proper, it has been shown that certain detergents and very common antiseptics, in subbacteriostatic concentrations, can acquire bactericidal power when used in combinations (12). Highly varying results have been reported of the simultaneous effect of antibiotics and detergents on different bacterial species, as shown by some observations listed in table 1.

### MATERIAL AND METHODS

The 32 strains of staphylococci may be classified by origin into two groups. Strains 1—23 had been isolated by Dr. M. Parmala from various hospital cases. The remaining 9 strains were isolated from patients at the Kiljava Sanatorium. Of these, 6 strains derived

TABLE 1

SOME EARLIER OBSERVATIONS ON THE COMBINED EFFECT OF ANTIBIOTICS AND SURFACE-ACTIVE AGENTS

| Bacterium                        | Surface-active Agent              | Nature of Combined Effect                                   |  |                 |
|----------------------------------|-----------------------------------|---|--|-----------------|
|                                  |                                   | Synergistic   | Indifferent  | Antagonistic    |
| <i>E. coli</i>                   | Salicain <sup>1</sup>             | Achromycin<br>Aureomycin<br>Chloromycetin<br>Penicillin (6) |  | Tyrothricin (6) |
| <i>E. coli</i>                   | Tween 80                          |   | Achromycin<br>Aureomycin<br>Chloromycetin<br>Penicillin (6)                      | Tyrothricin (5) |
| <i>Microc. flavus</i>            | Tween 80                          | Bacitracin (2)  |  |                 |
| <i>Microc. flavus</i>            | Tergitol 4                        |   |  | Bacitracin (2)  |
| <i>Microc. flavus</i>            | Benzethonium chloride             | Bacitracin (2)  | <sup>1</sup> ) Salicain = diaethylamino aethylester of para amino salicylic acid |                 |
| <i>Microc. pyog. var. aureus</i> | Sodium salt of dehydrocholic acid | Penicillin (10)   |  |                 |
| <i>Microc. pyog. var. aureus</i> | Tween 20                          | Penicillin (7)  |  |                 |
| <i>Mycobact. tuberculosis</i>    | Tween 20                          | Dihydrostreptomycin (7)                                     |  |                 |
| <i>Mycobact. tuberculosis</i>    | Tween 80                          | Chloromycetin (15)<br>Penicillin (8)<br>Streptomycin (3)    |  |                 |
| <i>Mycobact. tuberculosis</i>    | Triton A—20                       | Dihydrostreptomycin (13, 14, 9)                             |  |                 |

from different cutaneous or subcutaneous infections, two from pleural empyemas, and one from suppurative otitis media.

With a view to selection of surface-active agents, preliminary experiments were carried out with 11 detergents which are given and described in greater detail in the attached list.

## LIST OF DETERGENTS

## I Anion-active agents

- Rexol = triethanolamine salt of dodecyl benzene sulfonic acid (class I/F). Technical Products Ltd., London.
- SM-AH = sodium dioctyl sulfosuccinate (class I/E). Badische Anilin- & Soda-Fabrik A.G., Ludwigshafen a. Rhein.
- Teepol X = secondary sodium alkyl sulfate (class I/L). Technical Products Ltd., London.

## II Cation-active agents

- Sapamine A = oleyl amino diaethylamine acetate (class II/D).
- Sapamine L = " " " lactate (class II/D). Ciba A.G., Basle.

## III Nonionic agents

- Carbowax 6000 = polyethylene glycol mono oleate (class III/D) Glyco Products Co., Brooklyn, N.Y.
- D—O—R—9 = ethylene oxide condensation product of dodecyl alcohol (class III/B). Badische Anilin & Soda-Fabrik A.G., Ludwigshafen a. Rhein.
- Etoxol AP—19 = polyglycol ether of paraoctyl phenol (class III/D). A. Ahlström Oy., Varkaus (Finland).
- Nonisol 210 = polyoxyalkylene fatty ester (class III/B). Geigy Industrial Chemicals, Ardsley, N.Y.
- SM—O = ethyleneoxide condensation product of an alkyl phenol (class III/C). Badische Anilin- & Soda-Fabrik A.G., Ludwigshafen a. Rhein.
- Tween 20 = condensation product of ethylene oxide and sorbitan monolaurate (class III/B). Atlas Powder Co., Wilmington, Del.

It was shown in the preliminary experiments that all the anionic compounds (Rexol, SM-AH, Teepol X), as well as the two cationic (Sapamine A and L), in subbacteriostatic concentrations enhanced the effect of antibiotics on staphylococci. By contrast, the nonionic compounds proved fairly indifferent in the concentrations 1:10,000—1:100,000. In a few cases, using Etoxol AP-19 and Tween 20, an action differing from that of the antibiotic alone was demonstrated.

One agent of each group was included in the experimental series proper, and so low a concentration was chosen as to render it improbable for the detergent alone to exert any appreciable bacteriostatic action.

The agents selected and their concentrations were as follows:

|               |          |
|---------------|----------|
| I Teepol X    | 1:40,000 |
| II Sapamine L | 1:50,000 |
| III Tween 20  | 1:10,000 |

The following antibiotics were studied: penicillin, tetracycline, dihydrostreptomycin, neomycin, chloramphenicol, and polymyxin B. This last drug, itself not highly potent against the staphylococcus, was included for the purpose of finding out whether its potency would be increased by the addition of surface-active agents.

Bacto-sensitivity disks (Difco) were used in this study, and the concentrations were:

|                     |        |
|---------------------|--------|
| Penicillin          | 1 unit |
| Tetracycline        | 30 mcg |
| Dihydrostreptomycin | 10 mcg |
| Neomycin            | 30 mcg |
| Chloramphenicol     | 30 mcg |
| Polymyxin B         | 10 mcg |

#### Experimental series:

Saline solutions containing the above detergents in the rations given were prepared. To 9.5 ml of this solution was added 0.5 ml of a broth culture of bacteria kept for 24 hours in the incubator at 37°C. The surfaces of Levinthal agar plates were wetted with the suspension thus obtained, and the supernatant fluid was decanted. After about 15 minutes, antibiotic disks were placed on double dishes, three different disks on each.

Since the thickness of the agar can affect the accuracy of the method (4), flat-bottomed Petri dishes of equal size and equal amounts of agar were always used. The dishes were kept in the incubator for 18 to 24 hours, and the diameters of the inhibition zones were then measured with the accuracy of one millimetre. The mean of two double values was taken as the final result.

Similar series in which the staphylococcus suspension did not contain any detergent were prepared for control purposes. The possible action of the surface-active agent as such was studied by observing the growth of peripheral colonies.



TABLE 2

MEAN DIAMETER OF INHIBITION ZONE, MM.

| Group                  | Penicillin | Tetracycline | Dihydrostreptomycin | Neomycin | Chloramphenicol | Poly-myxin B |
|------------------------|------------|--------------|---------------------|----------|-----------------|--------------|
| Control                | 13.6       | 22.6         | 10.8                | 10.6     | 25.0            | 9.5          |
| Teepol X<br>1:40,000   | 20.7       | 31.1         | 13.9                | 12.8     | 29.4            | 9.5          |
| Sapamine L<br>1:50,000 | 20.3       | 30.8         | 14.2                | 12.0     | 29.4            | 10.1         |
| Tween 20<br>1:10,000   | 13.9       | 23.1         | 10.8                | 10.4     | 25.2            | 9.6          |

## RESULTS

The detergent dilution as such was found to have a bacteriostatic effect in the case of 4 out of the 32 strains studied (Teepol X in two and Sapamine L in two). The inhibition zone diameters obtained for the remaining 28 strains (not showing the above phenomenon) are given in table 2.

For the purpose of dealing statistically with the results, the effect of the detergent on each of the antibiotics and bacterial strains was calculated according to the following formula:  $d_2 - d_1 = \Delta$ , where  $d_2$  = diameter of inhibition zone in the presence of detergent, and  $d_1$  = corresponding diameter in control experiment. The following grouping by size of  $\Delta$  was obtained:

$$A: \quad \Delta > 5 \text{ mm}$$

$$B: \quad 5 \text{ mm} \geq \Delta > 1 \text{ mm}$$

$$C: \quad 1 \text{ mm} \geq \Delta \geq -1 \text{ mm}$$

$$D: \quad -1 \text{ mm} > \Delta \geq -5 \text{ mm}$$

$$E: \quad -5 \text{ mm} > \Delta$$

In groups A and B, thus, the potency of the antibiotic was greater in the presence of the detergent than without it. In group C there

TABLE 3  
CLASSIFICATION OF RESULTS ACCORDING TO RESPONSE OF STAPHYLOCOCCI IN  
THE PRESENCE OF DETERGENT

| Antibiotic          | Detergent                    | Combined Effect |    |             |              |   |   |
|---------------------|------------------------------|-----------------|----|-------------|--------------|---|---|
|                     |                              | Synergistic     |    | Indifferent | Antagonistic |   | X |
|                     |                              | A               | B  | C           | D            | E |   |
| Penicillin          | * Significance of Difference |                 |    |             |              |   |   |
|                     | Teepol X***                  | 23              | 3  | 1           | 2            | 1 | 2 |
|                     | Sapamine L***                | 20              | 6  | 1           | 3            | — | 2 |
|                     | Tween 20                     | —               | 10 | 16          | 6            | — | — |
| Tetracycline        | Teepol X***                  | 21              | 1  | 7           | —            | 1 | 2 |
|                     | Sapamine L***                | 19              | 5  | 6           | —            | — | 2 |
|                     | Tween 20                     | —               | 9  | 18          | 5            | — | — |
| Dihydrostreptomycin | Teepol X***                  | 5               | 9  | 15          | 1            | — | 2 |
|                     | Sapamine L***                | 10              | 5  | 14          | 1            | — | 2 |
|                     | Tween 20                     | —               | 2  | 24          | 6            | — | — |
| Neomycin            | Teepol X***                  | 2               | 20 | 7           | 1            | — | 2 |
|                     | Sapamine L***                | —               | 20 | 8           | 2            | — | 2 |
|                     | Tween 20*                    | —               | —  | 27          | 4            | 1 | — |
| Chloramphenicol     | Teepol X***                  | 10              | 15 | 4           | —            | 1 | 2 |
|                     | Sapamine L***                | 11              | 15 | 2           | 1            | 1 | 2 |
|                     | Tween 20                     | —               | 9  | 14          | 9            | — | — |
| Polymyxin B         | Teepol X                     | 1               | 6  | 12          | 10           | 1 | 2 |
|                     | Sapamine L                   | 2               | 8  | 11          | 9            | — | 2 |
|                     | Tween 20                     | —               | 4  | 25          | 3            | — | — |

X = No. of cases showing inhibition due to detergent.

was no distinct difference, and in groups D and E the detergent had an antagonizing effect.

Table 3 shows the distribution of the results according to the synergism or antagonism caused by the combined effect.

#### RESISTANCE AND SENSITIVITY

The 32 staphylococcus strains included 19 which were resistant to one or several antibiotics. The term resistance here denotes that a minimum inhibition zone of 0.5 mm did not appear round the antibiotic disk used, in other words the diameter measured was less than 7 mm.

TABLE 4

EFFECT OF SENSITIVITY ON THE OCCURRENCE OF SYNERGISTIC ACTION AGAINST STAPHYLOCOCCI

Cases with synergism/All cases

| Detergent                  | Resistant Strains<br>( $d < 7$ mm) | Moderately Sensitive Strains<br>( $d = 7-15$ mm) | Highly Sensitive Strains<br>( $d > 15$ mm) |
|----------------------------|------------------------------------|--|--|
| I Teepol X                 | 14/35 (40 %)                       | 43/74 (58 %)                                     | 60/71 (85 %)                               |
| II Sapamine L              | 14/35 (40 %)                       | 46/74 (62 %)                                     | 62/71 (87 %)                               |
| III Tween 20               | 4/36 (9 %)                         | 5/80 (6 %)                                       | 21/77 (27 %)                               |
| Mean value<br>I + II + III | 32/106 (30 %)                      | 94/228 (41 %)                                    | 143/219 (65 %)                             |
| Mean value I + II          | 28/70 (40 %)                       | 89/148 (60 %)                                    | 122/142 (86 %)                             |

 $d$  = diameter of inhibition zone on control dishes.

In table 4 the bacterial strains are classified into three sensitivity groups according to the diameter of the inhibition zone. In the groups thus obtained, the role of the synergistic effect was calculated and expressed as a percentage. The table shows that Teepol X and Sapamine L about equally often enhanced bacteriostatic action. Tween 20, by contrast, much more rarely caused enhancement of potency of the antibiotic. Using the method outlined above it was found that, the more sensitive the bacterial strain, the more frequently did synergism occur.

## DISCUSSION

The results of the experiments indicate that the anionic compound Teepol X and the cationic Sapamine L in subbacteriostatic concentrations enhanced the potency of penicillin, tetracycline, dihydrostreptomycin, neomycin and chloramphenicol in vitro. The activity of polymyxin B, however, which is fairly weak against staphylococci, did not seem to increase in the presence of detergents. The nonionic compound Tween 20 was not found to enhance the potency of the antibiotics. In the case of neomycin it sooner had an antagonizing effect.

The mechanism of the combined effect of antibacterial drugs and surface-active agents is a complicated question which is as yet largely unsolved (1, 2, 3, 10, 11, 15). For this reason care should be taken, as pointed out in some earlier reports (1, 11), not to combine these substances in clinical work without a previous laboratory examination.

#### SUMMARY

The combined effect of 6 different antibiotics and 3 surface-active agents on staphylococci of various types has been studied by the paper-disk assay, and the following results were obtained:

The anionic compound Teepol X, used in a subbacteriostatic concentration (1:40,000), increased the bacteriostatic action of penicillin, tetracycline, dihydrostreptomycin, neomycin and chloramphenicol. It did not affect the activity of polymyxin B.

The effect of the cationic compound Sapamine L (1:50,000) was exactly similar.

The nonionic Tween 20 (1:10,000) generally showed a fairly indifferent behaviour.

Synergism occurred more frequently when using sensitive strains than with resistant strains.

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## A POSSIBLE MECHANISM OF ACTION OF ETHYL ALCOHOL ON THE CENTRAL NERVOUS SYSTEM

by

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The physiological and biochemical mechanisms underlying the symptoms of acute ethyl alcohol intoxication have so far remained quite obscure. Since it is obvious that the main effect of alcohol is exerted on the central nervous system, much effort has been directed towards the elucidation of the action of alcohol on the metabolism of this organ. In studies with severely intoxicated men, Battey, Heyman and Patterson (1) found that the oxygen consumption of the brain was significantly lower than in the same subjects after the intoxication had subsided. This observation can be reproduced on an experimentally simpler level by a study of the oxygen consumption in slices of brain cortex tissue. Ghosh and Quastel (4) and Wallgren and Kulonen (12) have shown that if the respiration of rat brain cortex slices is measured under certain conditions, ethyl alcohol causes a significant drop in oxygen uptake. The conditions required to obtain this effect include stimulation of the respiration by either 0.1 M potassium chloride (4) or electrical impulses (12). The technique of stimulation by electrical impulses was originally introduced by McIlwain (5).

However, it has not been easy to explain the decrease in oxygen uptake in brain cortex tissue caused by alcohol. Beer and Quastel (2) have studied the effect of alcohol on the respiration of isolated brain mitochondria, and Truitt, Bell and Krantz (11) have investig-

ated the effects of alcohol on oxidative phosphorylation. Neither of these studies have revealed any effect of alcohol on these most fundamental mitochondrial reactions. Hence it has not been possible to explain the decreased oxygen consumption on the basis of simple inhibition of mitochondrial respiration.

At this point it may be illuminating to recall some mechanisms regulating the rate of mitochondrial respiration. It is obvious that an oxidizable substrate and oxygen are necessary requirements. On the other hand, the phosphorylation coupled to the oxidation imposes two other requirements: inorganic phosphate and a phosphate acceptor, usually adenosine diphosphate (ADP). The availability of the last mentioned component is largely dependent on the extent of the energy-consuming processes in the cell which require the breakdown of adenosine triphosphate (ATP) to ADP and inorganic phosphate. One specific energy-consuming and ATP-dependent function of the nerve cell is the transport of sodium ions across the cell membrane from the inside to the outside. When the cell conducts impulses, sodium ions enter it, and before the excitability of the cell can be re-established, sodium has to be moved to the outside again. In a conducting cell the ATP requirement is thus higher than in a resting one, and the increased splitting of ATP leads to the formation of more ADP, and ultimately to an increased respiration rate. The work of Mellwain (5) and his associates seems to have established that this is in fact the mechanism causing the increased respiration in brain cortex slices when these are stimulated by electrical impulses.

Ethyl alcohol causes a decrease in the electrically stimulated respiration of brain cortex slices, but does not directly affect the respiration of isolated mitochondria. A possible explanation for the inhibitory action of ethanol might be that it inhibits some process leading to the formation of ADP. It has now been possible to show, that microsomes isolated from brain tissue possess an adenosine triphosphatase activity which can be partially inhibited by alcohol in moderate concentrations (6—8).

#### METHODS

The isolation of the microsomal fraction and the assay procedures have been described elsewhere (7).

## RESULTS AND DISCUSSION

The ATP-ase activity of the microsomal fraction from brain tissue can be stimulated by the addition of sodium ions. This is illustrated in Table 1. Although the increase in activity is not large, it is very reproducible and appears to be specific for sodium. Potassium ions do not bring about a similar increase in activity.

TABLE 1

STIMULATION OF RAT BRAIN MICROSOMAL ATP-ASE BY SODIUM IONS. THE ASSAY MIXTURE CONTAINED: 0.1 ML OF MICROSOMAL SUSPENSION (THE MICROSOMES OF ONE RAT BRAIN WERE SUSPENDED IN 5 ML OF 0.25 M SUCROSE), 5MM ATP AS THETRIS SALT, 5 MM  $MgCl_2$  AND 20 MM TRIS-CHLORIDE BUFFER, pH 7.5. SODIUM IONS WERE ADDED AS NaCl, AND WHERE THEY WERE ABSENT, AN EQUIVALENT AMOUNT OF SUCROSE WAS ADDED TO MAKE THE MEDIUM ISOTONIC. THE FINAL VOLUME WAS 1 ML, THE INCUBATION TEMPERATURE 30°C AND TIME 10 MINUTES

| Exp. No. | Additions  | $\mu$ Moles $P_i$ Liberated |
|----------|------------|-----------------------------|
| 1        | None       | 0.56                        |
|          | 0.1 M NaCl | 0.75                        |
|          | 0.1 M NaCl | 0.77                        |
| 2        | None       | 0.44                        |
|          | 0.1 M KCl  | 0.43                        |
|          | 0.1 M NaCl | 0.57                        |

The characteristics of the sodium stimulation of the microsomal ATP-ase and its possible significance are discussed more fully elsewhere (7—8). For the purpose of the present discussion it is sufficient to say that the sodium stimulation is interpreted as a reflection of a reaction mechanism consisting of two coupled processes, one with sodium as substrate and the other with ATP. The reaction with ATP will not occur maximally unless sodium is present in the medium. This mechanism is considered to reflect the active transport of sodium across the cell membrane. Skou (10) reached similar conclusions in a study of the ATP-ase of submicroscopic particles derived from crab nerve. Recently, Post *et al.* (9) found an ATP-ase with similar properties in red blood cell membranes. Support for the view that the sodium stimulation of the ATP-ase activity implies the active transport of sodium has been obtained from inhibitor studies and is reported elsewhere (6—8).

TABLE 2

INHIBITION OF RAT BRAIN MICROSOMAL ATP-ASE BY ALCOHOLS. THE ASSAY CONDITIONS WERE AS DESCRIBED IN TABLE 1

| Exp. No. | Additions       | $\mu$ Moles $P_i$ Liberated    |                         |
|----------|-----------------|--------------------------------|-------------------------|
|          |                 | In Presence of<br>0.1 M $Na^+$ | In Absence<br>of $Na^+$ |
| 1        | None            | 0.86                           | 0.77                    |
|          | Ethanol 0.17 M  | 0.80                           | 0.68                    |
|          | Ethanol 0.08 M  | 0.81                           | 0.74                    |
| 2        | None            | 0.91                           | 0.80                    |
|          | Methanol 0.38 M | 0.84                           | 0.76                    |
|          | Ethanol 0.17 M  | 0.78                           | 0.72                    |
|          | Butanol 0.014 M | 0.85                           | 0.77                    |

Ethyl alcohol, as well as methyl and butyl alcohols, in concentrations causing a moderately severe intoxication, cause inhibition of both the unstimulated and the sodium stimulated ATP-ase (Table 2). It has been found, that two distinct types of inhibition of the ATP-ase occur, namely (a) a prevention of the stimulatory effect of sodium and (b) an inhibition which does not prevent stimulation by sodium. The alcohols cause an inhibition of the latter type, and therefore do not seem to be specifically involved in the mechanism of sodium stimulation. It is assumed that the alcohols, and also other inhibitors of the non-specific type interfere with the lipid structure of the membrane fragments in the microsomal fraction, and in this way with the rate of the ATP-splitting reaction.

Additional evidence in favour of the hypothesis that the effect of alcohol on the respiration of brain cortex slices is due to an effect on the cell membranes rather than to a direct inhibition of mitochondrial respiration, has been presented by Wallgren (13). After studying the effect of a number of inhibitors, he has reached the conclusion that inhibitors which are known to produce a depolarization of the nerve membrane show a synergistic effect with alcohol, whereas inhibitors which inhibit mitochondrial respiration directly will not show such a synergism. A similar synergistic action of inhibitors has also been observed with the microsomal ATP-ase. Thus, when alcohol and pyridine aldoxime



TABLE 3

SYNERGISM BETWEEN THE EFFECTS OF ETHANOL AND PYRIDINE ALDOXIME DODECYL IODIDE (PAD) ON THE MICROSOMAL ATP-ASE OF RAT BRAIN. CONDITIONS AS DESCRIBED IN TABLE 1

| Additions                                     | $\mu$ Moles $P_i$ Liberated  |                       |
|---|------------------------------|-----------------------|
|   | In Presence of<br>0.1 M NaCl | In Absence<br>of NaCl |
| None .....                                    | 0.85                         | 0.72                  |
| Ethanol 0.17 M .....                          | 0.76                         | 0.66                  |
| PAD, $3 \times 10^{-5}$ M .....               | 0.72                         | 0.71                  |
| Ethanol, 0.17 M and PAD, $3 \times 10^{-5}$ M | 0.62                         | 0.63                  |

dodecyl iodide (PAD), an inhibitor able to prevent the sodium stimulation of the ATP-ase, are added together, the inhibition caused by the two agents is additive (see Table 3). An additive inhibition by these two agents was also observed in brain cortex slices (13).

In view of these considerations the mechanism of action of alcohol on the nerve cells may be pictured as follows. Alcohol interferes with the active transport of sodium across the cell membranes, causing a decrease in its rate, which also means that the process that is the energy source of this reaction, the splitting of ATP, is slowed up. This leads to the production of less ADP and so through the mechanism of respiratory control, to a lower respiration rate.

The lower rate of sodium transport leads to a smaller difference in concentration on the two sides of the membrane, and thus to a lower membrane potential. This is in good agreement with observations on the effect of alcohol on the membrane potential of nerves (3). The lowering of the membrane potential leads at first to a slightly increased excitability but soon the excitability of the cell is decreased. The changes in the excitability of the nerve cells could provide a basis for the symptoms observed in alcoholic intoxication.

#### SUMMARY

The possible mechanisms of action of ethyl alcohol on the central nervous system are discussed. It is suggested that alcohol inhibits the mechanism of active sodium transport across nerve

cell membranes. The inhibition of the sodium-stimulated adenosine triphosphatase of brain microsomes by alcohol is demonstrated, and it is shown how this would provide an explanation of the various effects of alcohol on the intact nerve cell.

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## EFFECT OF AMMONIUM SULPHIDE ON THE HISTOCHEMICAL DEMONSTRATION OF CHOLINESTERASES WITH THE COPPERTHIOCHOLINE TECHNIQUE

by

ARTO PALKAMA

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Several studies have indicated that cholinesterases can be studied in a reproducible way with the aid of the histochemical method described by Koelle (3, 4) and simplified by Gomori (2). The method is based on enzymic hydrolysis of acetyl- or butyrylthiocholine in the presence of copper ions, which trap the liberated thiocholine in the form of a precipitate. The copper-containing colorless compound is made visible by treatment with ammonium sulphide, which forms brown copper sulphide in the sites of original enzyme activity.

In the course of a study of cholinesterases of the adrenal medulla (1) it was found that the results obtained were dependent on the last step of the procedure, *i.e.* treatment with ammonium sulphide. Therefore, the effect of varying concentration and length of time in ammonium sulphide was subjected to a systematic study.

### MATERIAL AND METHODS

White albino rats were used. The animals were decapitated with sharp scissors and the adrenals were fixed in calcium-formol for 2–4 hours. Fresh sections were cut at 40  $\mu$  with a freezing micro-

tome, floated on coverslips and dried. Thereafter the cholinesterases were demonstrated using Gomori's (2) simplified method. Acetylcholinesterase and non-specific cholinesterase were differentiated by using suitable substrate-inhibitor combinations (5, 1). After incubation the sections were rinsed 3 times for 5 min in 40% sodium sulphate solution at 37°C, treated with ammonium sulphide, washed in water and mounted in glycerol jelly. Eight per cent ammonium sulphide solution (May & Baker, Dagenham, England) was diluted to make 0.4%, 0.8%, 2.4%, and 8% solutions.

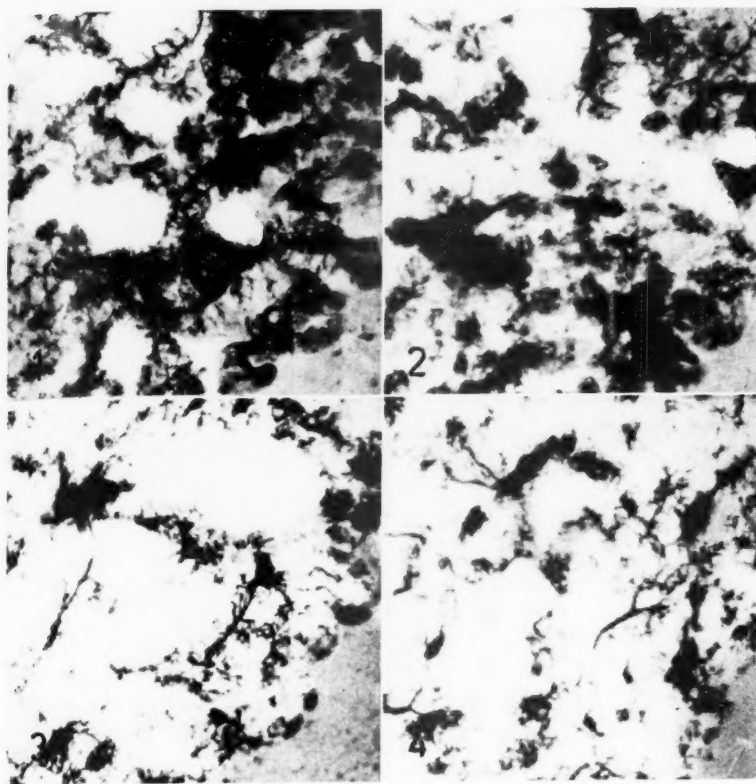
### RESULTS

The results are schematically presented in Table 1 and illustrated by Figs. 1—4. For the sake of simplicity, the intensity of the reaction was assessed in two types of structures only, *i.e.* the cytoplasm of the parenchymal cells and coarse nerve fibres. It can be seen that when the concentration of the ammonium sulphide was increased and/or the length of treatment in it prolonged, the intensity of the reactions in both the cytoplasm and the nerve fibres first weakened and finally disappeared altogether. At the same time, the yellow-brown color of the precipitate turned black in

TABLE 1

EFFECT OF TIME AND CONCENTRATION OF AMMONIUM SULPHIDE ON THE INTENSITY OF CHOLINESTERASE REACTION IN THE ADRENAL MEDULLA OF THE RAT

| Dilution    | Structure    | Time |       |      |      |      |       |       |
|-------------|--------------|------|-------|------|------|------|-------|-------|
|             |              | 5 s. | 30 s. | 1 m. | 2 m. | 4 m. | 10 m. | 24 h. |
| 1:20 (0.4%) | cytoplasm    | +    | +     | +    | +    | +    | ±     | ±     |
|             | nerve fibres | +++  | +++   | +++  | ++   | ++   | ++    | ++    |
| 1:10 (0.8%) | cytoplasm    | +    | +     | +    | +    | +    | ±     | ±     |
|             | nerve fibres | +++  | +++   | +++  | ++   | ++   | +     | +     |
| 4:10 (2.4%) | cytoplasm    | +    | +     | +    | ±    | ±    | —     | —     |
|             | nerve fibres | +++  | +++   | +++  | ++   | ++   | +     | +     |
| 1:1 (8%)    | cytoplasm    | +    | +     | +    | ±    | —    | —     | —     |
|             | nerve fibres | +++  | +++   | ++   | +    | ±    | —     | —     |



Figs. 1—4. — Non-specific cholinesterase in the adrenal medulla of the rat. The sections have been treated with 2.4% (4:10) ammonium sulphide solution. The section in Fig. 1 has been treated with ammonium sulphide for 5 sec., in Fig. 2 for 30 sec., in Fig. 3 for 2 min. and in Fig. 4 for 4 min. All the sections are from the same adrenal gland and the photomicrographs have been prepared exactly in the same way, as is evident from the equal gray tone in the cortical tissue, visible in the lower right corners of all figures. Note the gradual loss of the visible precipitate first from the cytoplasm and then from many nerve fibres.

Manification  $\times 130$ .

the persistently positive areas. The reaction in the cytoplasm disappeared first, the nerve fiber reaction in fine nerve fibres subsequently and last that in coarse fibres. It is likely that the first precipitated copper sulphide will gradually be transformed into a soluble copperammonium-complex in the ammonium sulphide solution. Reliable and reproducible results will be obtained when the sections are treated for 1 min in about 1% ammonium sulphide solution.

## SUMMARY

Increase of the immersion time in, and/or concentration of, ammonium sulphide in the thiocholine method for histochemical demonstration of cholinesterases results in a gradual loss of visible precipitate from positive areas. Treatment for 1 min in about 1% ammonium sulphide solution is recommended.

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## SKIN REACTIONS PRODUCED BY CERTAIN METALLIC SALTS<sup>1</sup>

by

ALI MUROMA

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This paper reports a study of the local reactions produced by metals in groups I, II and III of the periodic system of elements when injected into the skin of rabbits. The object was to determine whether their capacity to produce local reactions is correlated to their bactericidal and bacteriostatic activities, which are well known (1, 2, 3). The course of the reactions was also followed during 4 weeks from samples taken for histological examination.

A total of 36 metallic salts were used. They were dissolved in physiological saline, with the exception of salts of thallium and silver, which were dissolved in distilled water. The strongest solution used was 0.1 molar, from which other solutions were prepared by dilution 1:10. The solutions were injected intracutaneously into the depilated back of the animals, using about 20 injections into each rabbit. In reading the results, all clearly palpable papules were included and the mean diameter of each papule was measured.

### RESULTS

The lowest concentration producing within 24 hours a papule of minimum 5 mm diameter was first determined. For this purpose three parallel experiments were made with each solution and the mean value of the papules indicated the result. The results for the metallic salts used were as follows:

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<sup>1</sup> Aided by a grant from the Sigrid Jusélius Foundation.

|       |                 |  |
|-------|-----------------|--|
| 0.1   | molar solution: | H <sub>2</sub> AuCl <sub>4</sub> , Zn(C <sub>2</sub> H <sub>3</sub> O <sub>2</sub> ) <sub>2</sub> , BaCl <sub>2</sub> , GaCl <sub>3</sub> ;  |
| 0.01  | »               | : AgNO <sub>3</sub> , Be(NO <sub>3</sub> ) <sub>2</sub> , CdJ <sub>2</sub> , HgCl <sub>2</sub> , Al <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub>   |
| 0.001 | »               | : ScCl <sub>3</sub> , YCl <sub>3</sub> , InCl <sub>3</sub> , LaCl <sub>3</sub> , CeCl <sub>3</sub> , PrCl <sub>3</sub> ,<br>NdCl <sub>3</sub> , SmCl <sub>3</sub> , EuCl <sub>3</sub> , GdCl <sub>3</sub> , TbCl <sub>3</sub> , DyCl <sub>3</sub> ,<br>HcCl <sub>3</sub> , ErCl <sub>3</sub> , TmCl <sub>3</sub> , YbCl <sub>3</sub> , LuCl <sub>3</sub> . |

The other metallic salts studied, *i.e.*, LiCl, NaCl, KCl, CuSO<sub>4</sub>, RbCl, CsCl, MgSO<sub>4</sub>, CaCl<sub>2</sub>, Sr(NO<sub>3</sub>)<sub>2</sub>, Tl<sub>2</sub>SO<sub>4</sub>, produced in a concentration of 0.1 molar no reaction within 24 hours.

From each skin reaction a specimen was taken for histological examination.

These reactions, with the exception of those to CeCl<sub>3</sub>, NdCl<sub>3</sub>, EuCl<sub>3</sub>, TbCl<sub>3</sub>, HoCl<sub>3</sub> and LuCl<sub>3</sub>, were then followed during 4 weeks after injections into different rabbits. One rabbit was sacrificed 1, 3 and 4 weeks after injection and a histological specimen was taken of the reactions.

Histological examination revealed that the chlorides of all the rare earth metals (Sc, Y, La, Pr, Sm, Gd, Dy, Er, Tm and Yb) and of gallium produced in 1—4 weeks a granulomatous inflammation of the dermis, characterized by macrophages, lymphocytes, plasma cells, multinucleated foreign body giant cells, and occasionally giant cells of Langhans type. The other metallic salts studied produced only a transient inflammatory reaction, which disappeared almost completely within 4 weeks.

#### DISCUSSION

In comparing the skin reactions produced by metallic salts of groups I and II of the periodic system with their bacteriotoxic action, a certain degree of correlation is observed. A reaction was produced by the most toxic (1) of the group I metals, silver, in 0.01 molar solution, and by the next toxic, gold, in 0.1 molar solution, whereas other metals of this group produced no reaction. Likewise, the more toxic of the metals of group II, *i.e.*, Ba, Be, Zn, Cd and Hg, produced a reaction, in contrast to the less toxic Ca, Mg and Sr, which gave no skin reaction. In group III, the rare earth metals, whose bacteriostatic action has generally been found to be approximately equal but whose rate of bactericidal action varies (3), did not differ from each other in their capacity to produce skin reactions.



The observation that the rare earth metals and gallium produce a granulomatous inflammation in the dermis is interesting. This finding was not made in the case of the other metals studied. A similar granulomatous inflammation was observed by Steffee (4) following the intraperitoneal injection of certain rare earth metals into rats. He considered it possible that the reason for the development of granulomas is a precipitate formed when an acid solution of rare earth compounds is administered.

#### SUMMARY

Local reactions produced by the injection into the skin of rabbits of 36 metallic salts of groups I, II and III of the periodic system of elements were studied. When the capacity of the metals to produce reactions was compared with their bacteriotoxic action as presented in the literature, a certain degree of correlation was observed between these two actions.

Specimens for histological examination were taken from the skin reactions during 4 weeks. It was found that chlorides of the rare earth metals and gallium produced in 1—4 weeks a granulomatous inflammation, which was characterized by macrophages, lymphocytes, plasma cells, multinucleated giant cells, and occasionally giant cells of Langhans type.

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## THE ROLE AND ORIGIN OF THE FATTY MATERIAL IN CHALAZION

by

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(Received for publication April 24, 1961)

Though chalazion is fairly common in ophthalmic practice it has not so far been possible to clarify its etiology and pathogenesis (2). Chalazion is an inflammatory, chronic granuloma in the eyelid, usually attributed to the obstruction and infection of a meibomian gland (8). It is known that glandular obstruction alone is not the cause of chalazion (12) and that attempts to make cultures from chalazions are generally unsuccessful.

Chalazion is clinically a relatively indolent tumour with a comparatively poor record for spontaneous healing. Histopathologically chalazion must be considered to belong to the group of granulomatous inflammations. This group includes diseases of most heterogeneous etiology from tuberculosis to foreign body granuloma (3).

In the routine histopathological examination of 156 operatively removed chalazions the present writer's attention was attracted by the optically empty cavities commonly present in them. The inflammatory cell infiltrates seemed to be concentrated around these cavities, which led the authors to wonder if they might have a role in the pathogenesis of chalazion (Figs. 1, 2). It can be seen from the literature that these cavities, which usually appear to be empty optically in an ordinary histopathological preparation, have been assumed to contain fatty material deriving from dis-

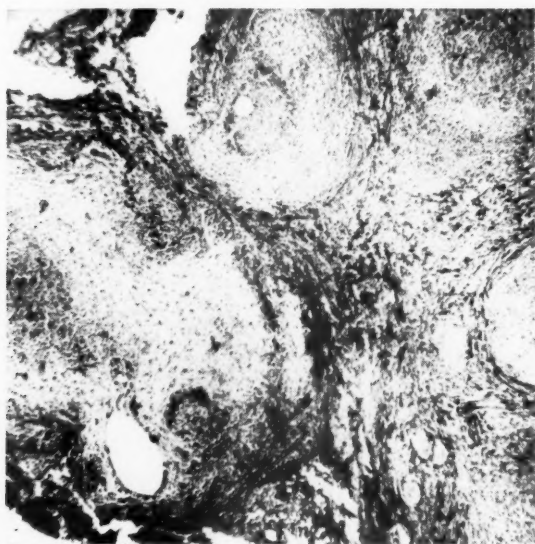


Fig. 1. — Photomicrograph of typical granulation tissue of the chalazion which consists of foci of varying size. An optically empty cavity formation is seen in the centre in two of them (x 36).

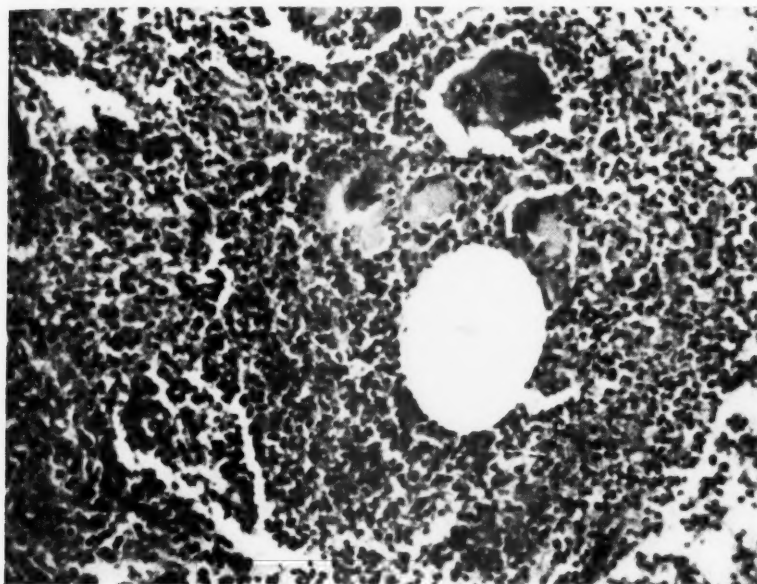


Fig. 2. — Photomicrograph of an optically empty cavity of the chalazion surrounded by dense inflammatory cell infiltration and several foreign body giant cells (x 175).

integrated meibomian gland but the fatty contents have disappeared during the technical treatment of the specimen (10).

Several workers, *e.g.* Schall (10) and Levaditi (7), have demonstrated fatty material in the granulation tissue of chalazion but only as tiny droplets in the histiocytes and in the tissue spaces around the cavity. Hagedoorn (4) seems to have been the first who demonstrated fatty material in the cavity itself.

A comparative study was performed in the present work between the fatty material encountered in the cavities of the granulation tissue and normal meibomian gland secretion.

#### MATERIAL AND METHODS

Frozen sections of both fresh and formalin-fixed material were made from surgically removed chalazions. In addition, normal meibomian glands were examined; they were obtained from eye patients in connection with other palpebral operations. The secretions were stained with fat stains: Scharlach red, Nile blue sulphate, and Sudan black B; Schultze's staining was performed according to Romieu for the demonstration of cholesterol, and Baker's modification of Smith—Dietrich's staining to elicit phospholipids. Double refraction was studied too. Furthermore, general stainings of the remaining part of the specimen after the frozen section had been taken were performed by v. Gieson's method. It was embedded in paraffin for this purpose.

In the majority of the cases in which optically empty cavities were found they were empty also in the frozen sections. In a part of them, however, Scharlach red positive material was retained inside the cavities and filled them almost completely. The colour was orange-red. Lipid stained in the same way was established admixed with inflammatory infiltrate around the cavities. It appeared there partly in the tissue spaces, partly phagocytised in the plasma of mononuclear histiocytes in droplet form. Nile blue sulphate stained the contents of the cavity a rose-purple-red, but the small extra- and intracellular droplets in the environment of the cavities did not take the stain. Sudan black B stained black both the cavity contents and the lipid droplets around it. Schultze—Romieu's staining elicited a negative cholesterol reaction in several repeat experiments. In the cavities there were intensely doubly

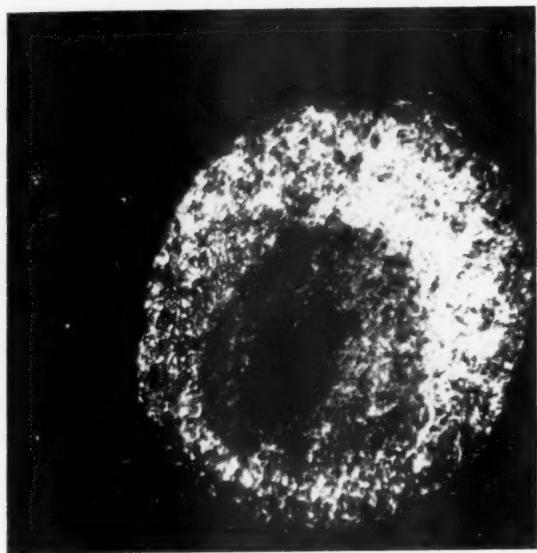


Fig. 3. — Photomicrograph of double refracting fatty material in the cavity of the chalazion (x 475).

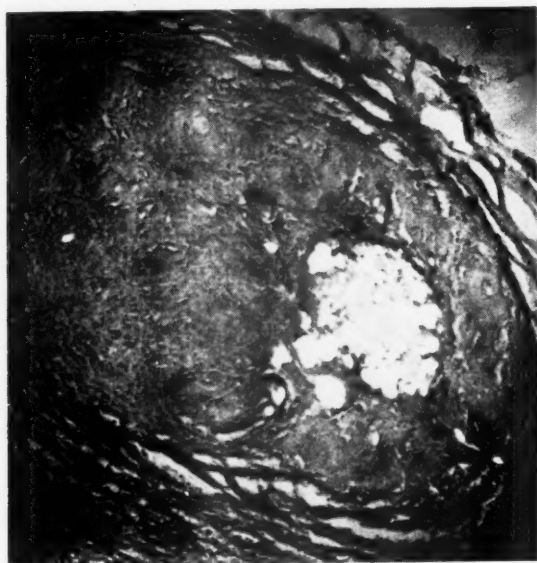


Fig. 4. — Photomicrograph of double refracting chrystals in chalazion surrounded with foreign body giant cells and histiocytes (x 95).

refractile crystal masses in a dense clump. The crystal size was so small that it was impossible to see the exact shape (Fig. 3). In the places with numerous multinuclear foreign body giant cells there were at the margin of the ragged cavities intensely doubly refractile, pine-needle shaped, long crystals (Fig. 4). The crystals were seen as optically empty slits in sections made from paraffin-embedded material. These crystals gave a faint Scharlach red positive reaction.

The secretion and epithelial cells inside a normal meibomian gland stained in lipid stainings like the contents of the chalazion cavity; Scharlach red gave an orange-red colour, Nile blue sulphate a purple-red and Sudan black B a black colour. The black was dense in the area of the «secretion», but of droplet form in the cell plasma. It was sparser towards the periphery of the gland cells. Schultze—Romieu's cholesterol reaction was negative. The meibomian gland «secretion» showed doubly refractile crystal masses morphologically identical with those in the cavities of the chalazion. It appeared obvious that the «secretion» of the meibomian gland and the contents of the chalazion cavities were at least to a great extent the same fatty material.

#### DISCUSSION

Although careful histopathological studies were made of chalazions in the 19th century (1, 6, 9, 13), the cavity formations in them scarcely attracted any attention earlier. Only Schall (10) studied these cavities more closely. He assumed that they contained fatty material probably deriving from retained meibomian gland secretion. Despite his frozen section technique, he was unable to establish the presence of lipids in the cavities. Schall also made animal experiments. By injecting vernix caseosa into rabbit ear lobe he succeeded in producing chalazion-resembling granulomas in them. The cavities in these granulomas were surrounded by inflammatory cells but were optically empty. There were small, fat-containing droplets in the inflammatory cells and tissue spaces. Schall was of the opinion that the fatty materials played an important role in the inflammatory tissue reaction of chalazion. After blocking of the duct of a meibomian gland, the further changes in a human chalazion probably result from chemical

irritation and destruction due to the blocked sebum or fat. Levaditi (7) injected the contents of a meibomian gland *e.g.* into the eyelid of monkey and produced a chalazion-resembling inflammatory granuloma. According to him, chalazion is »steatophagic granuloma» caused by phagocytosis of the secretion of a meibomian gland. Hagedoorn (4) expressed the belief that chalazion belongs to the large group of lipogranulomatoses like fat necrosis, encountered elsewhere in the body. He mentioned that cystic cavities occurred in fat necrosis and they also contained a fluid which resembled the soapy solution that ophthalmologists sometimes see oozing out of a chalazion. It was suggested by Hagedoorn that both chalazion and fat necrosis involve the effect of a lipolytic ferment.

It is common knowledge that cholesterol and fatty acids and other agents can cause granulomatous inflammation and the associated foreign body reaction. If chalazion is suspected to be due to retained fatty materials, there is reason to examine the histochemical reaction of the irritative influence of fat. Hirsch (5) made experimental studies of the effect of human fat, soaps and cholesterol on tissues. He came to the conclusion that at least three factors either singly or in combination contribute to the granulomatous reaction when the fatty materials irritate the tissues: (1) increase in acidity when the fat is hydrolysed, the hydrogen ion is concurrently released in the tissue fluid. Increase in acidity up to a certain level can cause necrosis in the tissue. (2) The nature of the soap compound formed in or about the oil phase during the chemical reaction between the fatty acid and the surrounding aqueous fluids of the tissues. Soaps slightly soluble or insoluble in water accumulate in the oil system or are precipitated in the tissue fluids, where inflammatory reactions occur which are dependent on the base in the soap compound. The soaps least soluble in the aqueous solutions apparently produce the maximal tissue reactions. (3) Such components of fatty materials as are completely insoluble in the aqueous solution and become crystallised when the soluble fats of the oil phase are transported into tissue fluids.

A chemical explanation of this kind, based on animal experiments, would seem to agree with the histological picture of chalazion and account for its chronic character. Chalazion contains crystals which are fatty materials, cavities containing fatty materials



and fatty phagocytosis around the cavities. The insolubility of the fatty materials, again, might explain the chronic course of the disease.

#### SUMMARY

The cavity formations encountered in chalazions were noted. These cavities, judging by an ample investigation material, seemed to play a central role in the chronic granulomatous inflammation of chalazion. The cavities contained fatty materials which gave the same staining and histochemical reactions as meibomian gland secretion. The cavities seemed to be filled with meibomian gland secretion, and this fat-containing, insoluble secretion mass might be the factor maintaining the inflammatory granuloma in chalazion.

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## METABOLIC AND ANTITHYROID EFFECTS OF PROLONGED ADMINISTRATION OF DINITROPHENOL IN MICE

by

KARI LAGERSPETZ and HARRI TARKKONEN

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According to fundamental laws of vital economics, the energy release in higher animals is regulated in accordance to their actual need of energy. It has become evident that the energy used by the animal is derived from catabolism through coupled synthesis of phosphates. The oxidative phosphorylation is the main method of adenosine triphosphate (ATP) production in aerobic organisms. The concept that the supplies of adenosine diphosphate (ADP) and inorganic phosphate usually control the rate of oxidation of substrates has been documented in a number of studies.

An especially powerful tool in the study of the oxidation-coupled biological trapping of energy is 2,4-dinitrophenol (DNP), the physiological effects of which were described by Magne, Mayer and Plantefol (15) and simultaneously by Cutting and Tainter (3). DNP was shown by Loomis and Lipmann (13) to be an uncoupler of oxidative phosphorylation. Later the concept was advanced, that the uncoupling was due to the stimulation of the breakdown of ATP (27). The strong induction of the ATP-ase activity by DNP and its mechanism was studied by Slater and Hülsmann (21) and by Löw (14). At relatively low concentrations, DNP obviously is a specific uncoupling agent, although it probably in higher concentrations also affects the actions of the respiratory chain (20). The stimulation of the oxygen consumption caused by the loss of respiratory control as a result of the action of DNP in various

systems has been the subject of a number of studies. The uncoupling effect of DNP has been studied by determining the P/O ratio in mitochondrial suspensions. Brody (2) reviewed the earlier literature on the subject.

Thus DNP belongs to the compounds whose immediate biochemical actions are relatively specific and well-known. Therefore, and also because its importance as an experimental tool in the studies of energy metabolism, it was considered interesting to find out, whether prolonged administration of DNP, thus causing a chronic partial uncoupling of the oxidative phosphorylation, would result in compensatory changes in the organism against the action of this compound. The oxygen consumption, R.Q., and colonic temperature were chosen as the physiological variables studied. In order to gain further insight on the effects of the prolonged administration of DNP, the structure of the adrenals, the liver and of the thyroid were studied and some determinations of the ATP content of the blood made.

#### EXPERIMENTAL

Thirty male 4 to 6 months old Swiss albino male mice (mean weight 30 g) were divided in lots of 10. The first group of animals received a single subcutaneous injection of 20 mg of DNP as sodium salt per kg body weight. The colonic temperatures of the animals were measured with a thermocouple inserted to the depth of 18 mm:s into the colon fifteen minutes before the injection and 15, 30, 60, and, in some cases, even 90 and 120 minutes after the injection. The rate of oxygen consumption and R.Q. were measured by the method of Haldane and Kendeigh, as described earlier (11). In order to eliminate the effects of the initial increase in the motility of the test animals in the animal chamber, the mice were kept in the animal chamber for 15 minutes before the actual period of measurement. The determinations of the oxygen consumption and R.Q. were made during the 15 minute periods at 30—15 minutes before the injection and at 15—30 and 45—60 minutes after it and, in some cases also later.

The second group of mice received 10 mg of DNP per kg twice daily for a period of 5 days. After this treatment the animals received a single dose of 20 mg of DNP per kg and their colonic

temperature, oxygen consumption and R.Q. were recorded before and after the injection, as described above.

The animals of the third group received 10 mg of DNP per kg twice daily for a period of 30 days. Subsequently, they were treated like the animals of the previous groups.

The results of these experiments are presented in Tables 1 to 3. They can be summarized thus: 1) The oxygen consumption of the animals is not significantly affected by the administration of DNP in small doses for 5 or 30 days. 2) The stimulation of the oxygen consumption caused by a single dose of DNP is significantly smaller in animals which have received small doses of DNP for 5 days than in normal animals ( $t = 2.12$ ,  $p = 0.05$ ). The pretreatment with DNP for 30 days even somewhat more decreases the effect of

TABLE 1

THE EFFECT OF A SINGLE DOSE (20 MG PER KG) DNP ON THE OXYGEN CONSUMPTION (ML/G/H) OF MALE MICE SUBJECTED FOR 5 OR 30 DAYS TO PRETREATMENT WITH TWO DAILY DOSES OF 10 MG DNP PER KG. EACH GROUP CONSISTS OF 9 ANIMALS. STANDARD ERROR IS SHOWN AT EACH MEAN.

| Pre-treatment | Oxygen Consumption (ml/g/h) |                 |             |             |              |
|---------------|-----------------------------|-----------------|-------------|-------------|--------------|
|               | Control                     | After Injection |             |             |              |
|               |                             | 15—30 min.      | 45—60 min.  | 75—90 min.  | 105—120 min. |
| none . . . .  | 4.78 ± 0.24                 | 7.58 ± 0.41     | 6.89 ± 0.24 | 6.51 ± 0.41 | 5.10 ± 0.37  |
| 5 days        |                             |                 |             |             |              |
| DNP . .       | 5.50 ± 0.25                 | 6.60 ± 0.18     | 6.07 ± 0.23 | —           | —            |
| 30 days       |                             |                 |             |             |              |
| DNP . .       | 5.36 ± 0.17                 | 6.14 ± 0.28     | 5.48 ± 0.12 | —           | —            |

TABLE 2

THE EFFECT OF A SINGLE DOSE DNP ON R.Q. OF MALE MICE SUBJECTED FOR 5 OR 30 DAYS TO PRETREATMENT WITH DNP. EACH GROUP CONSISTS OF 9 ANIMALS. STANDARD ERROR IS SHOWN AT EACH MEAN.

| Pre-treatment  | 100 × R.Q. |                 |            |            |              |
|----------------|------------|-----------------|------------|------------|--------------|
|                | Control    | After Injection |            |            |              |
|                |            | 15—30 min.      | 45—60 min. | 75—90 min. | 105—120 min. |
| none . . . . . | 79 ± 1     | 70 ± 2          | 72 ± 1     | 70 ± 2     | 78 ± 7       |
| 5 days DNP     | 81 ± 3     | 82 ± 3          | 83 ± 3     | —          | —            |
| 30 days        |            |                 |            |            |              |
| DNP . . . .    | 93 ± 3     | 92 ± 4          | 88 ± 4     | —          | —            |

TABLE 3

THE EFFECT OF A SINGLE DOSE OF DNP ON THE COLONIC TEMPERATURE OF MALE MICE SUBJECTED FOR 5 OR 30 DAYS TO PRETREATMENT WITH DNP. EACH GROUP CONSISTS OF 10 ANIMALS. STANDARD ERRORS VARY FROM 0.003 TO 0.01.

| Pre-treatment | Colonic Temperature °C |                 |         |         |         |          |
|---------------|------------------------|-----------------|---------|---------|---------|----------|
|               | Control                | After Injection |         |         |         |          |
|               |                        | 15 min.         | 30 min. | 60 min. | 90 min. | 120 min. |
| none ....     | 37.8                   | 38.1            | 38.0    | 37.8    | —       | —        |
| 5 days        |                        |                 |         |         |         |          |
| DNP ..        | 37.9                   | 38.2            | 38.1    | 37.8    | —       | —        |
| 30 days       |                        |                 |         |         |         |          |
| DNP ..        | 37.7                   | 38.0            | 38.2    | 38.0    | 37.7    | 37.8     |

DNP on the oxygen consumption, the difference between the means for the 5 days' group and for the 30 days' group being, however, not statistically secured ( $t = 1.61$ ,  $p < 0.15$ ). 3) The mean R.Q. values are significantly higher in animals which have received DNP in small doses for 5 days than in normal animals ( $t = 2.28$ ,  $p < 0.05$ ). The R.Q. is highest in the animals which have received DNP for 30 days, the difference of the means for the 5 days' and 30 days' groups being also significant ( $t = 2.45$ ,  $p < 0.05$ ). 4) A single dose of 20 mg of DNP per kg does not affect the R.Q. in any group of animals. 5) The colonic temperature level of the animals is not affected by the prolonged administration of DNP.

Seventeen Swiss albino male mice were treated in the following way: five animals received for 30 days twice daily subcutaneous injections of physiological saline (control group), five animals received twice daily an injection of 10 mg of DNP per kg for five days and seven animals were subjected to this treatment for 30 days. After this, the animals were decapitated and the thyroid removed with the underlying portion of the trachea and the larynx. Subsequently, the adrenals and a piece of the liver were removed and all tissues fixated in Bouin's fluid (Lillie's modification). Tissues were embedded in paraffin wax, cut in slices of 5 to 7  $\mu$  in thickness and stained with Weigert's iron hematoxylin and Domagk's thiazine red — picric acid solution or with Masson's thrichrome stain (the thyroids).

In order to demonstrate the possible differences in the functional state of the thyroid the following method was found satisfactory.

From each of four sections of each thyroid an area was drawn with a magnification of  $450 \times$  with the aid of Reichert's Visopan. The area taken by the thyroid colloid was measured twice with an Amsler planimeter. The mean percentage of the colloid area from the whole area studied was then calculated for each thyroid. This gives an approximate index for the relative colloid content of the thyroid. Uotila and Kannas (26) used volumes instead of areas in the calculation of the relative amount of colloid in the thyroid.

No histological differences were observed in the adrenals of the control and experimental groups. This is in accordance with the early observations of Schulte and Tainter (19, 22), who found that chronic DNP administration did not affect the adrenal structure of the rabbit or that of the dog.

The histological appearance of the liver was similar in animals of all groups. Schulte (18) did not find any evidence on an altered liver function or structure in dogs subjected to a prolonged treatment with DNP.

The relative amount of colloid in the thyroid for test animals is expressed in Table 4. From this it appears that the amount of colloid, measured as the areal percentage, was significantly higher than in controls only in animals treated for 30 days with DNP ( $t = 3.85$ ,  $p < 0.01$ ).

TABLE 4

THE EFFECT OF PROLONGED ADMINISTRATION OF DNP ON THE RELATIVE COLLOID CONTENT OF THE THYROID IN MALE MICE. THE PERCENTAGE OF THE AREA COVERED BY COLLOID IN SECTIONS EXAMINED IS PRESENTED. STANDARD ERROR IS SHOWN AT EACH MEAN.

| Daily Dose of DNP             | Saline         | $2 \times 10$ mg/kg | $2 \times 10$ mg/kg |
|-------------------------------|----------------|---------------------|---------------------|
| Duration of treatment         | 30 days        | 5 days              | 30 days             |
| Number of test animals        | 5              | 5                   | 7                   |
| Mean colloid percentage ..... | $40.8 \pm 3.2$ | $41.5 \pm 2.4$      | $57.4 \pm 2.8$      |

In addition, it was observed that these animals had a flattened glandular epithelium as well as slightly elongate nuclei (Fig. 2). The glandular epithelium of the control animals as well as that of those subjected only for 5 days to DNP was cuboidal with round nuclei (Fig. 1). The vascularization of the thyroid was less pronounced in animals treated for 30 days with DNP. According to

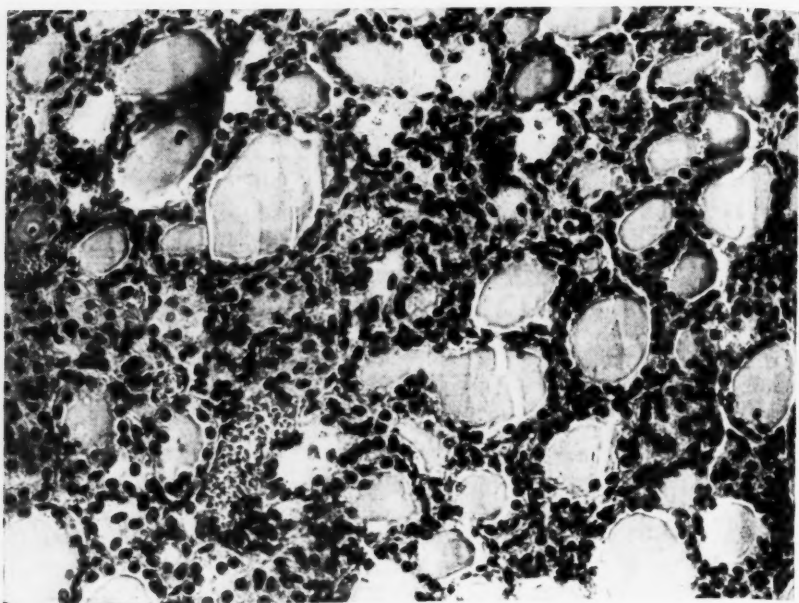


Fig. 1. — Photomicrograph of the thyroid of a saline-injected control mouse. Masson's trichrome,  $\times 300$ .

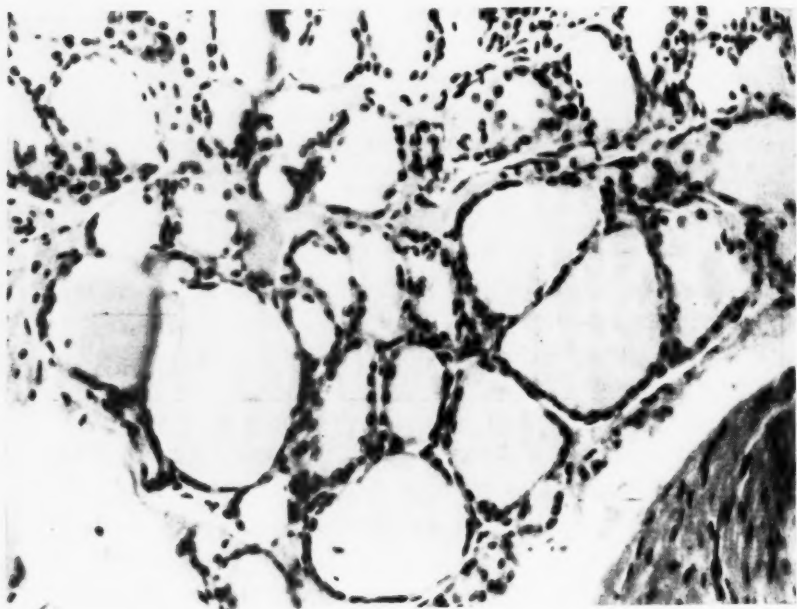


Fig. 2. — Photomicrograph of the thyroid of a mouse treated for 30 days with injections of DNP. Masson's trichrome,  $\times 300$ .



the present concept on the relations between the structure and the functional state of the thyroid (1, 23, 24, 25), the histological picture of the thyroids of the mice treated with DNP for 30 days indicates a marked hypofunction of the thyroid. Earlier studies have shown that the administration of 25 mg of DNP per kg every 8 hours intraperitoneally for 21 or 30 days or 0.15% DNP in the diet for 18 or 30 days does not cause a histological or weight change in the thyroid of rats although the plasma level of protein-bound iodine is markedly decreased (6, 28). In their studies on the thyroid-pituitary interrelationship, Goldberg, Wolff and Greep (8) gave chrySTALLINE DNP to rats 0.2% in their diet for 17 days. The thyroids of these animals were smaller and showed signs of hypofunction, when compared with the thyroids of the control animals.

#### DISCUSSION

The present experiments show that the stimulation of the oxygen consumption by DNP was smaller in animals which had received DNP earlier in smaller doses. The development of tolerance to the lethal and respiratory effects of DNP in dogs was observed already by Magne, Mayer and Plantefol (16). It was also found, that the excretion of glucuronic acid derivatives of DNP was enhanced when repeated doses of the compound was given to dogs (5). However, the somewhat better tolerance of the animals treated for 30 days with DNP in contrast to those treated for 5 days seems to indicate that other, slower mechanisms for the compensation of the stimulation of oxidation by DNP are also to be considered. It would be of interest to find out, whether the tolerance would appear in the metabolism of excised tissues and subcellular preparations.

Another result of the present experiments was the rise of R.Q. during prolonged DNP administration. As DNP enhances the ATP-ase activity in cells, the ATP concentration of tissues probably decreases. This was checked preliminarily by determining the ATP concentration of blood in six animals treated with DNP for 6 days and in five controls. The determination of ATP was performed according to the enzymatic test method of Bücher and Schuart, using the test combination manufactured by Boehringer. The concentration of ATP in the blood varied from 2.0 to 25.0 mg-%

(mean 13.0 mg-%) for the test animals and from 20.2 to 32.8 mg-% (mean 28.8 mg-%) in the controls, thus indicating a lower concentration of ATP and a greater variability in the level of ATP in the blood of animals which had received DNP for 6 days. The ATP of the blood is almost exclusively contained in blood corpuscles and in platelets. If this result is assumed to reflect the conditions in tissues in general, the oxidation of fats, which is dependent on the supply of ATP, may thus be inhibited by the prolonged DNP administration and the carbohydrate utilisation relatively enhanced (17). This would cause the observed rise in R.Q.

The enhancement of carbohydrate utilisation was observed already in the earliest studies on the effects of DNP (15). It was, however, later found that the principal substrate of the oxidation accelerated by a single non-lethal dose of DNP must be other than carbohydrate (10). The accelerated metabolism is not dependent on the enhanced utilisation of carbohydrates only. The fact that a single non-lethal dose of DNP did not affect the R.Q. of mice confirmed this result (11). Prolonged administration, however, appears to cause a relative shift in the substrate of oxidation to carbohydrates.

Apparent rise of R.Q. is observed when lethal doses of DNP are given to mice (11). This is a result of premortal anoxemia and acidosis, observed in rats and dogs after a high dose of DNP (10). With single non-fatal doses, none of these phenomena appear (10), the tendency being more to acapnia and alkalosis (9), which could cause an apparent decrease in R.Q., but not the progressive rise occurring during prolonged administration observed in the present study.

From the standpoint of vital economics, the decrease in the thyroid activity following prolonged administration of DNP is quite conceivable. Both thyroid hormone and DNP are considered to be uncouplers of the oxidative phosphorylation, although their mechanisms of uncoupling seem to be different (2). The results of uncoupling, an enhanced ATP-ase activity and an accelerated liberation of energy as heat with a looseness of the respiratory control, are similar to both compounds. Thus the administration of a non-physiological uncoupler, DNP, seems to cause a compensatory decrease in the level of the physiological uncoupler, the thyroid hormone. The mechanism of this compensation is not yet known. Goldberg, Wolff and Greep (8) did not observe any change



in the granulation of the beta-cells of the anterior hypophysis, which cells are conceived to be the site of the TSH production. Goldberg, Wolff and Greep arrived at the conclusion that the action of prolonged administration of DNP on the thyroid is not explainable on the basis of the classical feed-back theory of the thyroid control exerted by hypophysis (7, 8). So far, no satisfactory explanation of the action of DNP on the thyroid can be suggested. It must be pointed out, that the administration of DNP represents a heat load to the animal. This heat load is eliminated by the vasomotor mechanisms of the physical thermoregulation. In this respect, the administration of DNP is physiologically equivalent to the transference of the animal to a warm environment, which is also known to cause decrease in the thyroid activity (4, 8, 12).

#### SUMMARY

1. The oxygen consumption and colonic temperature of Swiss albino male mice is not altered by giving subcutaneous injections of 10 mg 2,4-dinitrophenol (DNP) per kg twice daily for 5 or 30 days.

2. R.Q. of the test animals increases progressively during the prolonged DNP administration. This may be due to the decreased ATP level of the tissues which decreases the utilization of fats.

3. The oxygen consumption of the test animals becomes progressively less liable to stimulation by a single dose of DNP.

4. No histological changes were found to occur in the adrenals or in the liver during the DNP treatment.

5. The thyroids of the animals treated for 30 days with DNP showed clear signs of hypofunction. The thyroids of the animals treated for 5 days only were similar to those of the control animals.

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## A METHOD FOR GETTING RID OF *TRICHOSOMOIDES* *CRASSICAUDA* IN LABORATORY RATS

by

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(Received for publication May 19, 1961)

*Trichosoimoides crassicauda* is a nematode parasite of the urinary tract of rats. It has been found in all the colonies of laboratory rats in Finland (8 colonies) (1), Denmark (2 colonies) and Sweden (1 colony) so far investigated. It has also been encountered in laboratory rats in Switzerland (5) and in the United States (6, 7) and thus seems to be commoner than is generally realised. Observations on wild rats indicate that in naturally infested populations larvae of the nematode may be expected to be circulating in tissues other than the urinary tract of the host for a large part of the life-span of the rat (2). The infection seems to be able to cause both blood and tissue eosinophilia (3) and it is possible that the larvae may also cause other tissue changes in the hosts. It thus seems advisable to try to get rid of these parasites in rats used for experimental work.

First an attempt was made to obtain rats free of the parasite by means of anthelmintics. The effectiveness of the drugs was mainly estimated by their influence on the number of nematode eggs passed with the urine of the rats. The attempt met with failure although one of the drugs used had some effect on the number of eggs in the urine of the host. Eventually, an uninfested colony of rats was produced by mating rats not passing nematode eggs in their urine and by using their offspring for further breeding.

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The urine of the rats was collected by means of glass funnels fitted beneath small round wire-floored cages. A spindle-shaped piece of glass, the smallest diameter of which exceeded the diameter of the tip of the funnel, was inserted between the latter and the collecting bottle. This allowed the urine to flow over its surface into the bottle, whereby contamination with faeces was avoided. The urine excreted between 4 p.m. and 9 a.m. was centrifuged and the sediment poured onto glass slides and examined microscopically for eggs. The mature golden-brown eggs of *Trichosomoides crassicauda* are spheroidal in shape and are capped at both ends with opercular plugs (Fig. 1). They are about  $65\ \mu$  long and thus are fairly easily observed even under low power magnification.

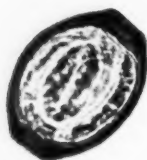


Fig. 1. — Mature, unstained egg of *Trichosomoides crassicauda*.  $\times 450$ .

Before the method is described by which an uninfested colony of rats was obtained, the results of the experiments with anthelmintics will be briefly dealt with.

*Hexylresorcinol* in doses of 40 mg in 1 ml of olive oil, was given by gastric instillation twice daily for 4 days to 9 infested rats. The treatment did not cause any decrease in the number of eggs passed in the urine. Since the dose of hexylresorcinol was fairly large, the attempt was regarded as a failure. 100 mg of *piperazine hydrate* given by gastric instillation once daily for 4 days to 5 rats did not have any influence on the number of eggs in the urine. Nor did tap water containing 1 per cent of this substance given as the sole source of drinking fluid to 6 rats for 5 days have any such effect.

Another group consisting of 6 rats was given 0.4 ml »Fuadin» (Bayer) i.m. once daily for 7 days. On the last day of the treatment the number of eggs in the urine had undergone a pronounced decrease. 6 days later, however, almost as many eggs were found in the urine of these rats as before the treatment. The results were so promising that a further group of 9 rats was given 0.4 ml »Fuadin» twice daily for 10 days. On the last day of the treatment very few eggs were found in the urine of these rats but, as in the first trial with »Fuadin» 6 days later the number had increased, and 1 month later nematodes were found in the bladders of the treated rats. The dose of the drug was so large that the general physical condition of the animals was affected. One of the rats in the last group did not pass eggs in its urine before treatment with »Fuadin». In spite of this nematodes were found in its bladder 1 month after the last dose. Since the findings of others (8) and personal observations indicate that one cannot expect mature parasites in the bladders of experimentally infected rats until about 7 weeks after the first eggs have been fed, it seems that »Fuadin» did not affect larvae circulating in the tissue of the host either.

In the attempt to get rid of *Trichosomoides crassicauda* by mating rats with no eggs in their urine and by breeding their offspring, full-grown rats were used at first. Among 87 females, 9 rats were found which did not pass eggs in their urine. These females were mated with males in whose urine eggs could not be found. The urine of the females was frequently inspected for eggs before partus. The litters were weaned at 18 days and the urine of the mothers was examined for the presence of eggs for a further 5 days. If no eggs were found, the offspring were regarded as uninfested. Nevertheless the urine of this first generation of supposedly uninfested rats was examined for eggs because the possibility of infection of the embryo *in utero* cannot be ruled out a priori — trichina cysts, for instance, have been found in the diaphragm of a child six weeks old (4). No eggs, however, were found in the urine of this first generation.

It is possible that the full-grown rats used above had some natural resistance to infection with *Trichosomoides crassicauda*. Since the uninfested strain was to be used for experiments with *Trichosomoides crassicauda*, the procedure was later somewhat modified. Rats of infested colonies generally start to pass eggs in their urine when weighing between 110 and 180 g. Thus instead of using older rats young females weighing about 90–100 g were mated and their urine was not examined for eggs until 18 days after partus when the litters were weaned. The percentage of females

passing eggs in their urine was considerably lower than when full-grown rats were used as above. Many of the young female rats seem to have been infected when mated, since several weeks later they started to pass eggs in their urine in spite of having been kept isolated. The colony of rats obtained by breeding the offspring was regarded as less different from the original stock of rats with respects to resistance to *Trichosomoides crassicauda* than the offspring of the older animals.

During this work the rats were kept in the same room as infected ones. Some precautions were taken, however, to avoid spread of the infection. In the animal room there were 6 rows of cages on top of each other. Infested rats were never kept in cages above the ones used for supposedly uninfested ones. The cages were washed thoroughly before being used for uninfested rats. Only hot water and detergents were used. The animals were taken care of by a person not usually in contact with infested rats. To avoid transmission of infection from one cage to another the attendant used smooth rubber gloves and rinsed his hands in running water between the handling of rats from different cages. Food for the supposedly uninfested rats was prepared and stored separately. The cages had wire-mesh floors, but as an added precaution the absorbent material used on the trays below the cages was taken from paper sacks not used for other animals and was discarded into separate boxes to avoid contamination of the trays. The floor of the room was washed frequently. Sweeping was avoided. In this way the rats were kept at the laboratory for 1.5 years without becoming infected as judged by the absence of eggs in the urine and of worms in the bladders of autopsied animals. The observations agree with the finding of Thomas (9) that uninfested rats may be kept in the same room as infested ones without becoming infected. The uninfested rats were later transferred to another laboratory where no other rats are kept. At present 5 generations of rats have been kept uninfested. Studies are in progress to reveal what pathological changes this nematode might cause in its host.

#### SUMMARY

A description is given of a method by which rats were freed from infection with *Trichosomoides crassicauda*. Precautions taken to keep the uninfested colony free from the infection are described.

An attempt to get rid of the parasites with some anthelmintics met with failure.

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## THE GLUCURONIDE CONJUGATION OF PHENOLPHTHALEIN IN MAN

by

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(Received for publication June 15, 1961)

It was stated in connection with the investigations on the glucuronide conjugation processes using phenolphthalein as glucuronidogenic substance in the animal experiments, that phenolphthalein excreted into the bile is solely in conjugated form, viz. phenolphthalein glucuronide. When administering phenolphthalein in small doses no excretion of free phenolphthalein into the bile or into the urine was detected (4, 5).

The present writers were unable to find any sulphate conjugation of phenolphthalein. This is in agreement with the previous findings of Di Somma (2). The absorption and excretion of phenolphthalein in man has been studied by Fantus and Dyniewicz (3) and Abel and Rowntree (1).

These writers found phenolphthalein after peroral administration in human urine partly in the conjugated and partly in the free form. The nature of the conjugate was not identified. A rather small part of administered phenolphthalein was excreted into the urine, and excretion was very slow. In human experiments they found only conjugated phenolphthalein in the urine when the doses were from 30 to 60 mg, while with larger doses (300 mg) free phenolphthalein was present in addition to the conjugate.

The previous studies on the absorption and excretion of phenolphthalein in cats (4, 5) has led the present writers to study these phenomena in humans.



## MATERIAL AND METHODS

The test persons were 12 male medical students (age 19—23 years), 11 patients of a military hospital (age 19—20 years) and female patients in the Surgical Clinic, University of Turku.<sup>1)</sup> The patients in the military hospital had suffered from mild upper respiratory infections and had been feverless at least two days. Their SR was  $< 15$  mm/hour. The surgical patients suffered from bile stones located in the gall bladder or in the common bile duct. The drainage of the bile into the gall bladder was undisturbed.

To the group of students and military hospital patients phenolphthalein (Merck *pro analysi*) was given perorally in gelatine capsules in the morning into the empty stomach. The dosage was 500 mg. Blood samples were taken with 3 hours intervals. Urine was collected during 12 hours and 24 hours after the ingestion of drug.

In the group of the surgical patients also the bile was collected during the operation. The collection of bile took place about six hours after the administration of phenolphthalein. All the samples were analysed for phenolphthalein before and after hydrolysis with  $\beta$ -glucuronidase. The details of the technique are described in a previous report (5).

The nature of the conjugate was chromatographically identified using phenolphthalein-mono- $\beta$ -glucuronic acid (Sigma Chemical Co) as the reference substance. The chromatographic method is described in a previous work (5).

## RESULTS AND DISCUSSION

The free form of phenolphthalein was not detectable in blood, urine, or bile. The conjugated form of phenolphthalein in bile and urine was identified to be phenolphthalein glucuronide ( $rf=0.19$ ). It was resistant to alkali hydrolysis and presented thus a glucuronide conjugate of ether type.

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1) The writers are greatly indebted to Dr. Olli Klossner for the collection of bile samples.

The amount of phenolphthalein glucuronide excreted into the urine during 12 hours was small varying from 0.4 per cent to 0.8 per cent, mean 0.5 per cent, of administered dose. The 24 hours excretion varied from 0.7 per cent to 12 per cent, mean 5.3 per cent. All the results are calculated as free phenolphthalein. All test persons excreted detectable amounts of phenolphthalein glucuronide into the urine.

The blood concentrations of phenolphthalein were very small, detectable amounts were found only in 3 persons of 23. The highest concentration, observed 3 hours after ingestion, was 0.17 mg/100 ml whole blood. In all the other samples the concentration was lower than 0.1 mg/100 ml whole blood, which is the lowest sensitivity limit of the method employed.

The total excretion into the bile was impossible to determine because the drainage of the bile into the intestine was free. The concentration of phenolphthalein glucuronide in bile was 17.3 mg/100 ml bile. It is to be seen that the resorbed amount of phenolphthalein is small. A very great deal of resorbed phenolphthalein was excreted into the bile as glucuronide conjugate. The nature of the phenolphthalein conjugate in blood was not identifiable due to the small concentrations. The absorption of phenolphthalein and its conjugation with glucuronic acid was found to be identical with the animal experiments. The intensive excretion of phenolphthalein into the bile in disagreement with the prevailing concept that phenolphthalein is in a very small degree absorbed from the intestinal tract of man (3).

The cathartic action of phenolphthalein was not noted with this dosage (500 mg). This agrees with previous findings (3).

#### SUMMARY

The absorption and excretion of phenolphthalein, a glucuronidogenic substance, was studied in humans by peroral loading. The free form of the compound was not detectable in blood, urine, or bile. The conjugated form was identified to be phenolphthalein glucuronide, which is very rapidly excreted in large concentrations into the bile. The blood concentrations were small, and the excretion into the urine was scanty.

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(in press).

## EFFECT OF PHENACETIN ON THE SERUM POTASSIUM AND THE FUNCTION OF THE PARATHYROID GLAND<sup>1</sup>

AN INVESTIGATION ON MEN AND RATS OF TWO SUSPECTED EFFECT  
MECHANISMS OF PHENACETIN ON THE KIDNEYS

by

ANTERO KASANEN, HEIKKI A. SALMI and UNTO VUOPALA

(Received for publication June 7, 1961)

The daily use of phenacetin is very common. In Denmark, 29.4 per cent of 698 hospital patients took drugs containing phenacetin daily (51). The comparable percentage for Finland was 20.7 (3). In 33.2 per cent of the phenacetin consumers the kidney function was reduced; the percentage was only 8.6 in a control group (5). Renal diseases were established in 13 per cent of patients consuming no phenacetin against 85 per cent for patients who had taken over 5 kg of phenacetin (3).

Of the pathological changes established the principal one was interstitial nephritis involving diffuse dilatation in the connective tissue and subsequent constriction of the tubuli and the capillaries of the interstitium. The basal membranes of the tubuli were thickened. Diffuse nephrocalcinosis could supervene later (13). The same changes have been produced experimentally by giving test animals phenacetin and concurrent injections of coli bacteria (7).

The effect mechanism of phenacetin-induced renal damage is still obscure, which led the present authors to consider two possible ways in which phenacetin might affect the kidneys. They followed the serum potassium and the function of the parathyroid gland in man and rat.

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<sup>1</sup>Aided by a grant from the Sigrid Jusélius Foundation.

*Effect of Phenacetin on the Serum Potassium.* — It has been shown previously that prolonged potassium deficiency leads to permanent and irreversible kidney lesions (4, 11). The pathological forms of changes are chronic pyelonephritis and interstitial fibrosis, and it is possible that as a result of the prolonged metabolic stress of potassium deficiency the kidney is more susceptible to bacterial infection (2).

These changes resemble histologically the alterations caused by phenacetin in the kidneys. As regards localisation, the changes occur in the same areas also in the inner medullary zone and the papillae. Phenacetin users also show an increased susceptibility to urinary tract infections (6, 12). The overuse of laxatives, for instance, is known to result in prolonged potassium deficiency (11), and the present writers suggest that this mechanism is also involved in the effect exerted by phenacetin.

The serum potassium of phenacetin abusers who had ingested over 1 g of phenacetin daily for several years, but in whom no signs of azotemia were demonstrable, was determined. The mean for 26 patients was 17.2 mg%, no different from the normal value.

Sixteen rats were given the following amounts of phenacetin during the experimental period of 5 weeks: week I 0 g, week II 0.75 g/kg daily, week III 0.37 g/kg daily, week IV 0.18 g/kg daily and week V 0.37 g/kg daily. The phenacetin was administered to the rats by tube once in 24 hours. Because it is insoluble in water, it was suspended in a tragacanth-water solution (0.5 per cent). The suspension contained 50 mg/ml of phenacetin. The test animals, 16 in all, like those of the control group, 15 in all, were kept on a standard diet (2).

No differences were established in the serum potassium values between the experimental group and the controls either at the beginning or at the end of the experiment.

*Effect of Phenacetin on the Function of the Parathyroid Gland.* — It is common knowledge that a great many renal changes are associated with hyperparathyroidism. The commonest renal changes in primary hyperparathyroidism are urinary calculi and recurrent or chronic urinary tract infection (8). The calculi are mostly small and symmetrical, and calcification can sometimes remain at the stage of diffuse nephrocalcinosis. On the other hand, it is known that the function of the parathyroid gland changes

secondarily in some renal diseases and, in addition, a changed calcium metabolism can cause renal osteopathy. Chronic interstitial nephritis and pyelonephritis are such diseases, in other words the same diseases as are produced by excessive ingestion of phenacetin. Vitamin D and PAS can cause changes in the function of the parathyroid gland (11) and the present workers consequently suspect that phenacetin might exert an effect along the same route. On the other hand, polyuria and polydipsia are also early symptoms in both conditions.

The present writers therefore determined the clearance of tubular absorption of phosphorus in 29 patients who had consumed an average of 1.6 kg of phenacetin during their life but whose renal function was nevertheless still normal. The TRP was calculated according to Chambers *et al.* (1) from the following formula:

$$\text{TRP} = 1 - \frac{\text{Phosphate clearance}}{\text{Creatinine clearance}}$$

$$\% \text{ TRP} = \left( - \frac{\text{Urine PO}_4 \cdot \text{Serum creatinine}}{\text{Urine creatinine} \cdot \text{serum PO}_4} \right) \cdot 100$$

The normal range of TRP is 78—90 per cent

The results were as follows:

|                          | Number of Patients | % TRP          |
|--------------------------|--------------------|----------------|
| Controls .....           | 15                 | 85.9 $\pm$ 3.2 |
| Phenacetin abusers ..... | 29                 | 82.3 $\pm$ 4.6 |

No differences were established.

The serum calcium was also normal, 9.4 mg<sup>100</sup>.

The parathyroid glands were weighed after the course of medication in the rat-man group and in the control group. The combined dry weight of both parathyroid glands was 0.72 mg and in the phenacetin group 0.68 mg on an average.

There was thus nothing indicative of hyperparathyroidism.

#### DISCUSSION

It was clearly demonstrable clinically that the incidence of certain renal diseases was higher among phenacetin consumers than in the control group. Such diseases of the kidney are papillitis

necroticans, chronic interstitial nephritis and pyelonephritis. There was no correlation between the serum potassium and the function of the parathyroid gland, and the genesis of these injuries. The question of the mechanism of effect has still to be settled, but phenacetin obviously causes direct biochemical changes in the connective tissue and papillae of the kidneys. These changes predispose to infections and they tend to have a more malignant course. The point can probably be resolved histochemically.

#### SUMMARY

To study phenacetin-induced renal changes in both men and test animals, the serum potassium and the function of the parathyroid gland were followed by means of serum calcium and %TRP determinations. Although both hypopotassemia and hyperparathyroidism display in the kidneys similar changes to those established in phenacetin abusers, phenacetin affected neither the serum potassium nor the function of the parathyroid gland.

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## EFFECT OF ASCORBIC ACID ON THE ABSORPTION OF POTASSIUM PHENOXYMETHYL PENICILLIN

by

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The absorption of oral penicillins has been studied by Herold *et al* (1). In their reports the serum potassium phenoxymethyl penicillin levels were markedly higher after simultaneously given ascorbic acid. A similar effect was not observed with other penicillins or other salts of phenoxymethyl penicillin, nor could they explain their results.

Since this observation might have a considerable clinical significance an attempt was made to find out if their result could be reproduced. These trials are reported in this paper.

### MATERIAL AND METHODS

The study was conducted with 9 male patients having no heart or renal failure, the ages varying 20—68 years. The patients fasted over night and at least 3 hours after having taken the drug.

Potassium phenoxymethyl penicillin was used as powder and manufactured by the pharmaceutical manufacturers Lääke Oy.

The patients were divided into two groups: one received 500 000 i.u. of penicillin and the other 500 000 i.u. of penicillin combined with simultaneously given 500 mg of liquid ascorbic acid. After an interval of one day the groups received the doses inversed.

The blood specimens were withdrawn 1, 2, 4, 6 and 8 hours after the intake of the drug. One sample was withdrawn before the



TABLE

SERUM PENICILLIN LEVELS (I.U./ML) AFTER THE ADMINISTRATION OF ONE ORAL DOSE OF 500 000 U. PHENOXYMETHYL PENICILLIN (VP) AND AFTER ONE ORAL DOSE OF 500 000 U. PHENOXYMETHYL PENICILLIN COMBINED WITH 500 MG ASCORBIC ACID (VP+C)

| Subject | Agents | Hours after Administration |      |      |      |      |
|---------|--------|----------------------------|------|------|------|------|
|         |        | 1                          | 2    | 4    | 6    | 8    |
| 1       | VP     | 2.2                        | 0.7  | 0.12 | 0    | 0    |
|         | VP + C | 2.8                        | 0.7  | 0.14 | 0    | 0    |
| 2       | VP     | 3.2                        | 1.2  | 0.29 | 0.14 | 0    |
|         | VP + C | 3.1                        | 1.2  | 0.25 | 0    | 0    |
| 3       | VP     | 3.3                        | 1.8  | 0.33 | 0.14 | 0    |
|         | VP + C | ≈ 5                        | 2.4  | 0.5  | 0.12 | 0    |
| 4       | VP     | 2.3                        | 0.88 | 0.13 | 0    | 0    |
|         | VP + C | 2.8                        | 0.78 | —    | 0    | 0    |
| 5       | VP     | 3.5                        | 1.2  | 0.12 | 0    | 0    |
|         | VP + C | 4.4                        | 1.4  | 0.18 | 0    | 0    |
| 6       | VP     | 14                         | 4.7  | 0.98 | 0.23 | 0.12 |
|         | VP + C | 8.4                        | ≈ 4  | 0.66 | 0.21 | 0    |
| 7       | VP     | 3.5                        | 0.7  | 0.19 | 0    | 0    |
|         | VP + C | 3.4                        | 1.0  | 0.19 | —    | 0    |
| 8       | VP     | 3.9                        | 1.1  | 0.16 | 0    | 0    |
|         | VP + C | 2.6                        | 1.3  | 0.32 | 0.17 | 0    |
| 9       | VP     | 2.6                        | 0.82 | 0.32 | 0.16 | 0.13 |
|         | VP + C | 2.8                        | 2.2  | 0.37 | 0    | 0    |

intake to determine whether the sera inhibited the test bacterial strain. The samples were centrifuged after the withdrawal and stored in an ice-box. The penicillin level determinations were performed by the «agar cup» method using a test strain *Sarcina lutea* 9341 (2).

## RESULTS AND CONCLUSIONS

The results are given in the accompanying table. No effect of ascorbic acid on the serum potassium phenoxymethyl penicillin levels were detected.

## SUMMARY

Serum penicillin concentrations have been studied in 9 men after doses of 500 000 i.u. potassium phenoxymethyl penicillin alone and combined with 500 mg simultaneously given liquid ascorbic acid. The serum penicillin levels were determined 1, 2, 4, 6 and 8 hours after the intake of the drug. Ascorbic acid had no effect on the absorption of potassium phenoxymethyl penicillin.

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## THE HEMAGGLUTININS AND THEIR INHIBITORS IN THE SEEDS OF *VICIA CRACCA*

by

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(Received for publication May 23, 1961)

In a previous paper (1) dealing with the development of hemagglutinins in *Vicia cracca* seeds we reported on the occurrence of inhibitors in the extracts of small green seeds for the agglutinins of the ripe seeds of the same species. At the stage in the development of the seeds when the inhibitors disappeared, the agglutinins appeared almost in their full strength. After this stage the seeds seemed to be capable to germinate.

Being interested in the extracts of the small green seeds we wanted to study if the extract would inhibit also the growth of the root tip.

### METHOD

The ripe seeds were slightly scarified to accelerate the germination. When the primary root was 5—20 mm long, the seeds were transferred upon a thin cork plate with small holes. The root was inserted through the hole and the plate was placed floating on the standard mineral solution (2). After 24 hours the length of the root was measured again. The gain of length given in the tables below is the mean value of 200 roots measured.

## MATERIAL

The green seeds of *Vicia cracca* were sieved through a set of sieves, and the different fractions were dried at room temperature. The seeds were ground immediately before the experiment, and extracted with 500 parts of water for one hour at 37 C°. These extracts were diluted 1+1 with the standard mineral solution (double strength).

## RESULTS

Table 1 shows the gain in length of the root and the presence of agglutinins and of inhibitors of agglutinins in the appropriate fraction of seeds extracted. These were: were 1) normal ripe seeds, 2) big green seeds, picked out by a sieve of 4.0 meshes, 3) small green seeds, picked out by a sieve of 2.0 meshes.

TABLE 1

THE GAIN OF THE LENGTH OF THE ROOT OF *VICIA CRACCA* GROWN IN EXTRACTS OF DIFFERENT SEED FRACTIONS

|                                | Extracts of |            |                             |                               |
|--------------------------------|-------------|------------|-----------------------------|-------------------------------|
|                                | Control     | Ripe Seeds | Big Green Seeds<br>Mesh 4.0 | Small Green Seeds<br>Mesh 2.0 |
| Average gain in length .....   | 4.9 mm      | 3.8 mm     | 4.0 mm                      | 1.5 mm                        |
| Agglutinins .....              |             | present    | present                     | none                          |
| Inhibitors of agglutinins .... |             | none       | none                        | present                       |

TABLE 2

THE GAIN OF THE LENGTH OF THE ROOT OF *VICIA CRACCA* GROWN IN EXTRACTS OF DIFFERENT SEED FRACTIONS

|                                | Extracts of |                         |                          |                          |
|--------------------------------|-------------|-------------------------|--------------------------|--------------------------|
|                                | Control     | Green Seeds<br>Mesh 4.0 | Green Seeds<br>Mesh 2.83 | Green Seeds<br>Mesh 2.38 |
| Average gain in length .....   | 5.2 mm      | 3.4 mm                  | 2.8 mm                   | 2.0 mm                   |
| Agglutinins .....              |             | present                 | present                  | none                     |
| Inhibitors of agglutinins .... |             | none                    | none                     | none                     |

The extract of small green seeds was strongly inhibiting. The roots grown in that extract turned their colour dirty brown. The extracts of big seeds also prevented the growth but slightly.

The results of a second and similar experiment are given in Table 2.

Apparently all the seed extracts inhibited some of the growth of the root. The amount of the inhibitor was maximal in non-agglutinating small seeds (mesh 2.0 and 2.38). As soon as the agglutinins were present, the growth inhibition seemed to diminish considerably.

As N-acetylgalactosamine, melibiose and raffinose inhibit the agglutinins of *Vicia cracca* (3) we tested whether these sugars could also inhibit the growth of the root of the plant. Table 3 shows that these sugars in 0.2 per cent concentration in the standard mineral solution did not inhibit the growth. However, galactose a poor inhibitor of the agglutinins inhibited already in this concentration the growth.

TABLE 3

AVERAGE GAIN IN LENGTH (IN MM) OF THE ROOT OF *VICIA CRACCA* IN 0.2 PER CENT SUGAR SOLUTIONS

|                             | Experiment I | Experiment II |
|-----------------------------|--------------|---------------|
| Glucose .....               | 4.2          |               |
| Galactose .....             | 3.2          |               |
| Melibiose .....             | 4.9          |               |
| Raffinose .....             | 4.6          |               |
| N-acetylgalactosamine ..... |              | 5.4           |
| Control .....               | 4.4          | 5.6           |

## SUMMARY

The smallest green seeds of *Vicia cracca* contain inhibitors of agglutinins and inhibitors of growth of the plant.

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## GEOGRAPHICAL DISTRIBUTION OF DIPHASIC TICK-BORNE ENCEPHALITIS IN FINLAND<sup>1</sup>

by

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(Received for publication June 28, 1961)

In the Åland archipelago, south-west Finland, diphasic tick-borne encephalitis was shown to be endemic in 1956 (4). In 1959 three strains of viruses of the tick-borne encephalitis complex were isolated from ticks collected on Kumlinge island in this region (8) and according to an epidemiologic antibody survey using the hemagglutination inhibition technic (10) antibodies to these group of viruses were present in about 13 per cent of the population in the archipelago (11). The first clinical cases of diphasic meningo-encephalitis on the mainland of Finland were recognized in 1957 (7). These cases were from the Lappeenranta region in eastern Finland. To study the probable distribution of tick-borne encephalitis viruses in this region ticks and blood samples from human beings and domestic animals were collected during summer 1958 in the eastern part of Finland<sup>3</sup>. In addition, blood samples from reindeer and domestic animals were collected in Lapland in winter 1959. The results of these two surveys will be discussed in this paper.

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<sup>2</sup> We wish to express our sincere thanks to Dr. and Mrs. Brian Holcombe who assisted in the collection of the blood samples and to Miss Christina Öhman who collected the ticks. The skilful technical assistance of Mrs. Saara Henriksen and Miss Aili Vehviläinen is also gratefully acknowledged.

<sup>3</sup> This sampling was made possible thanks to the kind assistance of the State Medical Board and the Finnish Red Cross.



meritime, the longest interval from bleeding to arrival at the laboratory was 3 to 4 days. The inadequate storage during summertime may have lowered the antibody titers of at least some sera.

*Collection of Information.* — A questionnaire concerning personal data and history of previous hospitalization and possible tick bites was filled in by all the blood donors. They were also asked about diseases that probably resembled diphasic meningo-encephalitis. For domestic animals data about age, sex, birthplace, ticks, (other ectoparasites), name, and address of the owner were collected.

*Collection of Ticks.* — In 1958, in July, 191 engorged and 620 fasting ticks were collected from 9 rural communes. Next year, in June, 620 engorged and 58 fasting ticks were collected from Uukuniemi, a rural commune on the Russian frontier in the south-east. By courtesy of Dr. Harald Johnson, Berkeley, California, U.S.A., some of the ticks were sent for classification to Dr. Glenn M. Kohls, Hamilton, Montana, U.S.A. They were all classified as belonging to the species *Ixodes ricinus*. Ticks were stored at room temperature in rubber stoppered tubes containing a piece of wet cotton wool. The rubber stoppers were opened daily. When ticks showed signs of dying, pools were immediately prepared from approximately 10 to 40 fasting adult ticks, 10 to 20 engorged ticks, 30 to 50 nymphs and 100 larval ticks. If pools could not be injected after preparation they were stored at  $-60^{\circ}\text{C}$  in an electric deepfreezer.

*Tissue Culture Methods.* — The following modification of a previously described method (3) was used. HeLa cells were grown in a medium containing 40 per cent pooled, inactivated (at  $56^{\circ}\text{C}$  for 30 min.), ultrafiltered human serum in Hanks' salt solution with 100 IU of penicillin, 100  $\mu\text{g}$  of streptomycin and 25 IU of mycostatin. Stock suspension of HeLa cells were made by trypsinization after counting the cells in a Hemocytometer 30,000 cells were distributed in tissue culture tubes ( $16 \times 160$  mm) in the above-mentioned growth medium. Rubber stoppered tubes were incubated for 3 to 4 days at  $37^{\circ}\text{C}$ . The tubes were washed three times with Hanks' solution and the cells were maintained in a medium consisting of 10 per cent, inactivated, antibody free calf serum in a modified Parker 199 solution without vitamins, purines, pyrimidines, riboses or cholesterol.

*Virus Strains.* — The louping ill virus used was obtained through the courtesy of Dr. G. ff. Edward, The Wellcome Research Laboratories, Langley Court, Beckenham, Kent, England, in 1955. It was used in tissue culture tests in its 23rd to 27th HeLa cell passage in this laboratory and in its 15th intracerebral mouse passage in neutralization tests in mice. The tissue culture virus was prepared in HeLa cells grown in Carrell flasks by inoculating louping ill virus into the maintenance medium described above. The tissue culture fluid was harvested 3 to 5 days after inoculation of the virus, distributed in lots of 1–2 ml in Wasserman tubes. The tubes were closed with rubber stoppers and stored at  $-60^{\circ}\text{C}$ . A separate tube was used for each test.



Mouse brain virus for neutralization was prepared according to W. McD. Hammon (2).

*Neutralization Test.* — All the sera were screened in a dilution of 1 to 4. 0.25 ml of serum was mixed with an equal volume of virus dilution containing 100 to 1,000 CPE<sub>50</sub> units of tissue culture virus. The virus was previously titrated in tenfold dilutions using 5 tissue culture tubes per dilution. The CPE<sub>50</sub> was calculated according to Reed and Muench (9). The virus-serum mixtures were incubated at 37°C for 2 hours, and 0.2 ml was inoculated into 2 tissue culture tubes. After 5 to 7 days of incubation at 37°C the results were read microscopically. A virus titration, a neutralization test with positive and negative serum, the virus dilution used in the neutralization test and occasionally serum in the dilution 1 to 4 without virus were included as controls in each test. Owing to the shortage of mice, the screening of the neutralizing capacity of sera was mainly done in mice with one virus dilution containing about 100–1,000 LD<sub>50</sub> units of virus which was mixed with an equal volume of undiluted serum. The virus-serum mixtures were incubated at 37°C for 60 minutes and 0.03 ml was inoculated intracerebrally into 3 to 5 white mice weighing 10 to 12 g. The mice were kept 14 days after inoculation. Deaths occurring within 5 days of the inoculation were not taken into account. Neutralization indices were determined in some cases by mixing undiluted serum with equal volumes of ten-fold virus dilutions beginning from 1 to 50,000. After incubation for 60 min. at 37°C the serum virus mixtures were inoculated intracerebrally in 0.03 ml amounts, using 5 mice per dilution.

*Virus Isolation Experiments.* — 0.01 ml of pools prepared as described were inoculated intracerebrally into one litter of suckling mice 1 to 6 days of age. The mice were observed for 14 days and second passages were made as follows: the mice were sacrificed, the brains removed aseptically, weighed and ground in a mortar and suspended in 10 per cent inactivated antibody-free rabbit serum in phosphate buffered saline to which 500 IU of penicillin and 500 µg of streptomycin per ml was added. In negative cases two blind passages were made.

## RESULTS

*Incidence of Neutralizing Antibodies against Louping Ill Virus in Human Sera Collected in East Finland.* — Out of the 1235 human sera, 13 neutralized louping ill virus in tissue culture. Ten of the positive sera were from healthy subjects and 3 from persons with a previous history of serous meningitis. Of the 22 persons with a previous history of encephalitis or meningitis (Table 1), all the sera from the encephalitis cases were negative when tested either in tissue culture or in mice against 100 LD<sub>50</sub> units.

The 3 positive cases of serous meningitis were from Uukuniemi and Saari, 2 rural communes near the Russian frontier. The cases

TABLE 1

RESULTS OF SERUM NEUTRALIZATION TESTS IN HELA CELLS AND MICE OF ENCEPHALITIS AND MENINGITIS CASES FROM EASTERN FINLAND

| Serum No. | Age | Sex    | Commune      | Diagnosis and Date of Illness                          | Neutralization                                     |   |
|-----------|-----|--------|--------------|--|--|---|
|           |     |        |              |  | Tested against 100 CPE <sub>50</sub> in HeLa Cells | Tested against 100 LD <sub>50</sub> in Mice |
| 2001      | 33  | Female | Lauritsala   | Encephalitis ac. virosa<br>September 1957              | < 4  | negative                                    |
| 2002      | 3   | "      | Luumäki      | Meningitis virosa<br>February 1957                     | < 4  | negative                                    |
| 2005      | 3   | "      | Lappeenranta | Meningoencephalitis<br>May 1958                        | < 4  | negative                                    |
| 2011      | 35  | Male   | Luumäki      | Encephalomeningitis ac. virosa<br>October 1957         | < 4  | negative                                    |
| 2036      | 11  | Female | Lemi         | Meningitis virosa<br>May 1957                          | < 4  | negative                                    |
| 2041      | 31  | "      | Lappeenranta | Encephalomeningitis virosa<br>December 1956            | < 4  | negative                                    |
| 2053      | 55  | Male   | Joutseno     | Encephalomeningitis ac.<br>January 1958                | < 4  | negative                                    |
| 2055      | 28  | "      | "            | Encephalomeningitis post<br>influenzae<br>January 1958 | < 4  | negative                                    |
| 2057      | 28  | "      | "            | Encephalomeningitis ac.<br>March 1957                  | < 4  | negative                                    |
| 2082      | 27  | Female | Imatra       | Encephalomeningitis virosa<br>April 1957               | < 4  | negative                                    |
| 2083      | 46  | Male   | "            | Encephalitis ac. post<br>influenzae<br>February 1958   | < 4  | negative                                    |
| 2084      | 19  | Female | "            | Meningitis serosa<br>June 1957                         | < 4  | negative                                    |

(Continued)

TABLE 1 (Continued)

| Serum No. | Age | Sex    | Commune        | Diagnosis and Date of Illness                       | Neutralization                                     |   |
|-----------|-----|--------|----------------|---|--|---|
|           |     |        |                |   | Tested against 100 CPE <sub>50</sub> in HeLa Cells | Tested against 100 LD <sub>50</sub> in Mice |
| 2098      | 13  | Male   | Ruokolahti     | Encephalitis virosa<br>November 1957                | < 4  | negative                                    |
| 2168      | 5   | "      | Parikkala      | Meningitis serosa<br>1954                           | < 4  | negative                                    |
| 2292      | 45  | "      | Joroinen       | Meningitis<br>July 1958                             | < 4  | negative                                    |
| 2340      | 59  | Female | Uukuniemi      | Meningitis serosa<br>August 1957                    | 128  | positive                                    |
|           | 35  | "      | "              | Meningitis serosa<br>August 1957                    | 64   | positive                                    |
| 2355      | 59  | Male   | Saari          | Meningitis serosa<br>September 1957                 | 64   | positive                                    |
| 2532      | 7   | "      | Rääkkylä       | Meningitis e<br>causa non<br>indicata<br>March 1955 | < 4  | negative                                    |
| 2551      | 4   | "      | Joensuu        | Meningitis lymphocytaria<br>Autumn 1955             | < 4  | negative                                    |
| 2580      | 15  | "      | "              | Meningitis serosa<br>March 1954                     | < 4  | negative                                    |
| 3035      | 18  | Female | Pieksämäen mlk | Meningitis<br>June 1956                             | < 4  | negative                                    |

from Uukuniemi were from the same family. Blood samples were later taken also from other members of the family, but they were negative. The elder female had a neutralization index of 10.000 when tested against louping ill virus in mice.

Five out of the 10 positive sera from healthy subjects neutralized 100 CPE<sub>50</sub> only in a 1 to 4 dilution and gave a negative result

in mice (Table 2). The other 5 had somewhat higher titres, up to 1 to 64. It is interesting to note that 2 of these sera collected in 1959 were from a family in which one positive case of serous meningitis had occurred (No. 2355, Table 1). The distribution of positive human sera is shown in Fig. 2.



Fig. 2. — Location of louping ill positive human (▲) and animal (●) sera in East and North Finland.

*Incidence of Neutralizing Antibodies against Louping Ill Virus in Domestic Sera Collected in East and North Finland.* — Out of 387 domestic sera 16 neutralized louping ill virus in tissue culture. Four sera had low titres (1 to 4) and 4 had titres of 1 to 128 or higher (Table 3). The latter also neutralized 400 LD<sub>50</sub> units of louping

TABLE 2

RESULTS OF NEUTRALIZATION TESTS IN HELA CELLS OF POSITIVE HUMAN SERA FROM EASTERN FINLAND

| Serum No. | Year | Sex    | Age in Years | Locality   | Neutralization Titre in HeLa Cells |
|-----------|------|--------|--------------|------------|------------------------------------|
| 2020      | 1958 | Female | 12           | Luumäki    | 16                                 |
| 2021      | "    | Male   | 58           | "          | 4                                  |
| 2028      | "    | Female | 75           | Lauritsala | 16                                 |
|           | 1959 | "      | 54           | Saari      | 64                                 |
|           | "    | Male   | 15           | "          | 16                                 |
| 2605      | "    | "      | 36           | Eno        | 8                                  |
| 2747      | "    | "      | 56           | Värtsilä   | 4                                  |
| 2823      | "    | "      | 24           | Nuijamaa   | 4                                  |
| 2880      | "    | "      | 29           | Miehikkälä | 4                                  |
| 3130      | "    | Female | 28           | Anttola    | 4                                  |

ill virus in mice. Three sera from sheep were also positive, but with lower titres (1 to 4, 1 to 16). These were negative when tested against 400 to 1,000 LD<sub>50</sub> units in mice. The geographical location of the positive domestic sera is shown in Fig. 2.

From North Finland only 2 positive cow sera were found, giving neutralizing titres of 1 to 32 and 1 to 16 (in tissue culture) respectively. These 2 sera were from the rural communes of Tervola and Pello, close to the Swedish frontier. The former serum gave partial neutralization also against 400 LD<sub>50</sub> in mice. The neutralization index of this serum against louping ill virus in mice was 80. The cow in question was borne in Tervola on 15th November, 1953. The parents of the cow came from South Finland. According to the same inquiry, ticks have never been seen either on cows or in nature in Tervola.

*Virus Isolation Experiments from Ixodes Ricinus Ticks.* — A total of 1489 ticks was used for the isolation experiments. The ticks were collected from the localities shown in Fig. 3. Several attempts were made to isolate virus from localities where positive human or animal sera had been found. One pool from Ukuniemi and one from Simpele caused disease in infant mice. All the mice inoculated were affected and some died within 14 days; no paralyses were observed. The brains were removed immediately. Sterility tests failed to elicit any bacterial contamination, and 10 per cent

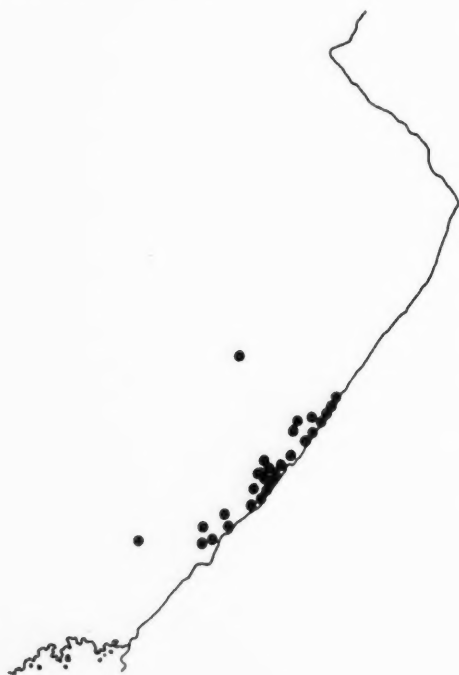


Fig. 3. — Location of tick sampling points in East Finland.

brain suspensions were inoculated undiluted for second passages in infant and adult mice both intracerebrally and intraperitoneally. The infant mice died in 10 days in both cases. The adult mice did not show any signs of illness. Further passages are being made to identify the agents isolated.

#### DISCUSSION

The recognition of the endemic occurrence of diphasic tickborne encephalitis in the Åland archipelago, south-west Finland (4), prompted a search for other endemic regions. The fact that tick-borne encephalitis was recognized in the Leningrad region in the Soviet Union already in 1949 (1) focussed attention on areas bordering the Russian frontier in the East of Finland. The results reported now and summarized in Table 4 show that tick-borne encephalitis viruses obviously are present also in this region.

TABLE 3

RESULTS OF NEUTRALIZATION TESTS IN HELA CELLS AND MICE OF POSITIVE DOMESTIC ANIMAL SERA FROM EASTERN AND NORTHERN FINLAND

| Serum No. | Species | Age, Years | Locality          | Neutralization                                     |  |
|-----------|---------|------------|-------------------|--|--|
|           |         |            |                   | Tested against 100 CPE <sub>50</sub> in HeLa Cells | Tested against 400 to 1,000 LD <sub>50</sub> in Mice |
| 5036      | Cow     | 6          | Lemi              | 4  | —  |
| 5054      | "       | 6          | Joutseno          | 4  | negative   |
| 5057      | "       | 2          | "                 | 16   | negative   |
| 5061      | Sheep   | 1          | Imatra            | 8  | negative   |
| 5072      | "       | 2          | Rautjärvi         | 4  | negative   |
| 5080      | Cow     | 3          | Simpele           | 256  | positive   |
| 5082      | "       | 5          | "                 | > 128  | positive   |
| 5084      | "       | 5          | Parikkala         | 16   | negative   |
| 5092      | "       | 8          | Ruokolahti        | 4  | negative   |
| 5093      | "       | 15         | "                 | 8  | negative   |
| 5163      | "       | 10         | Uukuniemi         | 128  | positive   |
| 5164      | "       | 14         | "                 | 256  | positive   |
| 5169      | Sheep   | 2          | "                 | 16   | positive   |
| 5184      | Cow     | 7          | Mikkelin mlk      | 16   | negative   |
| 5308      | "       | 6          | Tervola (Lapland) | 32   | positive*)   |
| 5324      | "       | 3          | Pello (Lapland)   | 16   | negative   |

\*) Neutralization index 80.

TABLE 4

INCIDENCE OF NEUTRALIZING ANTIBODIES AGAINST LOUPING ILL VIRUS IN HUMAN SERA AND SERA FROM DOMESTIC ANIMALS COLLECTED FROM DIFFERENT PARTS OF THE COUNTRY

| Date and Place of Sampling | Sera              | Total Number | Number Positive | Per Cent Positive |
|----------------------------|-------------------|--------------|-----------------|-------------------|
| 1958<br>East Finland       | Human             | 1,235        |                 |                   |
|                            | Encephalitis      | 10           | —               |                   |
|                            | Meningitis serosa | 12           | 3               | 25                |
|                            | Healthy subjects  | 1,213        | 10              | 0.8               |
|                            | Domestic animals  | 202          |                 |                   |
|                            | Cows              | 127          | 11              | 8.8               |
|                            | Sheep             | 51           | 3               | 5.9               |
|                            | Horses            | 23           | —               |                   |
|                            | Goats             | 1            | —               |                   |
| 1959<br>Lapland            | Domestic animals  | 185          |                 |                   |
|                            | Cows              | 75           | 2               | 2.7               |
|                            | Horses            | 9            | —               |                   |
|                            | Reindeer          | 101          | —               |                   |

The frequency of tick-borne encephalitis in this part of the country seems, however, to differ significantly from that in the Åland archipelago. The incidence of antibodies against this group of viruses among human beings seems to be only about one tenth of the corresponding incidence of antibodies in the highly endemic Åland region (11). Although the incidence of antibodies is clearly higher in domestic animals than in human beings, the tendency seems to be the same because the incidence in some endemic regions in the Åland archipelago is very high in cows and sheep (5). These findings confirm the repeatedly stressed fact that cows are good indicators of the presence of tick-borne encephalitis virus in nature. They also show, however, that tick-borne encephalitis is much less widespread in the lake district in East Finland than in the southwestern archipelago.

The cases of encephalitis and meningitis are too few to warrant any conclusions, especially since the diagnoses are retrospective and only 8 out of the 22 cases occurred during the period June—October when ticks are prevalent. But the fact that the 3 positive cases occurred during this period supports the diagnosis of tick-borne encephalitis. If these 3 cases are taken to be cases of diphasic tick-borne meningo-encephalitis, and bearing in mind the few cases diagnosed in 1957 (7), it may be concluded that sporadic but apparently infrequent cases of the disease occur also in this part of the country. This explanation concurs with the incidence of antibodies and is supported by previous statements that human cases occur in higher numbers only in regions where the virus has accumulated in nature. For this part of the country, this may be the case with the Uukuniemi and Saari regions where the 3 cases occurred and where several positive cow sera also were found.

The results of the study in Lapland are, however, somewhat difficult of interpretation. If, as has been assumed (13), ticks do not occur in this region, one would expect all the sera to be negative. In fact 2 positive cow sera were found. This seems to correspond to the incidence of louping ill virus antibodies in cattle in Sweden (12) and could probably be explained in one of the following ways: The cows in question contracted the infection in some other part of the country; ticks do occur in this region and transmit tick-borne encephalitis; tick-borne encephalitis viruses are transmitted by other vectors than ticks in this region; the neutralizing effect of



these two sera is due to antibodies against another type B group virus or is nonspecific.

As far as can be ascertained, at least one of the cows has not been outside the district in question, which seems to rule out the first alternative. A decision regarding the other possible explanations requires further studies. If tick-borne encephalitis occurs in Lapland, however, the frequency seems to be very low compared with the other regions studied to date.

It seems desirable to have the results of the immunologic studies confirmed by isolation of the virus from ticks collected in these regions. The two agents pathogenic for infant mice do not fulfill the criteria of tick-borne encephalitis viruses. The fact that similar agents have been isolated from ticks collected in the highly endemic Åland region, however, raises the question whether they represent variants of the «true» tickborne encephalitis viruses or some other hitherto unknown viruses and, finally, concerning their possible importance as a cause of human disease.

From the present and earlier studies it seems obvious that diphasic tick-borne encephalitis viruses are present at least in the south-west and in the south-east of the country. There are signs, however, (6) that this group of viruses may occur also in other parts of the country. This point and the nature of the unidentified viruses isolated from ticks requires further studies which are now in progress.

#### SUMMARY

Neutralizing antibodies against louping ill virus were demonstrated in human sera from healthy subjects and persons with a history of serous meningitis as well as in sera from domestic animals collected in East Finland, suggesting the presence of the tick-borne encephalitis complex of viruses also in this part of the country.

From ticks collected in this region 2 agents pathogenic for infant but not for adult mice were isolated. The nature of these agents remains obscure.

Neutralizing antibodies against louping ill virus were likewise demonstrated in 2 cow sera collected in Lapland where the occurrence of ticks has not been proved.

The importance of the above findings and the true distribution of this group of viruses in Finland is discussed.

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## THE INDIRECT EFFECT OF SHORT-DISTANCE X-RAY IRRADIATION ON THE MITOTIC ACTIVITY IN SOME ORGANS OF RATS<sup>1</sup>

by

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Fluctuations in mitotic activity of the skin provoked by roentgen ray irradiation have been obtained earlier in rats not only in the area treated but also outside it on the skin (7). An increase in the mitotic index was established in the untreated areas as soon as after 6 hours. This increase was attributed to the remote effect of irradiation which is transferred either humorally or via the blood to the environment. An endeavour was made in another work to elucidate the influence of blood irradiated *in vitro* on the mitotic activity of various organs by re-injecting the irradiated blood into the test animal<sup>8</sup>. However, no significant variation was established in the mitotic index. Teir (5) irradiated one external orbital gland of rats and found that there was an increase in the mitotic rate after one day and a fall after one week not only in this gland but also in the other.

The purpose of this investigation is to study whether locally and superficially effective irradiation has a distance effect not only on the homologous but also on the heterologous organs and their mitotic activity.

### MATERIAL AND METHODS

Two different experiments were made, using 3-month old white male rats of Sprague Dawley strain. The rats were kept 12—16

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<sup>1</sup> This work was supported by the Sigrid Jusélius Foundation.

animals in each cage. They weighed 150—200 g. In the first experiment 24 rats were divided into 4 groups and given short-distance x-irradiation with the Siemens-Monopan equipment of the Department of Radiotherapy, the Central Hospital (60 kV, distance 1 ½ cm., radiation field 2 sq.m. and treatment-time 43 seconds).

The treatment was administered at 3 p.m. on three successive days to the same area of the interscapular skin of the animals in such a manner that the skin to the left of the midline was pulled as much as possible outward from the body, the mouth of the tube touching this skin, so that x-rays could not reach other parts of the body. The single dose was 1,000 r and the total dose 3,000 r. The 16 rats serving as controls in the three groups (in one group only 4 rats) received no irradiation.

Six hours after termination of irradiation treatment the 6 rats of the first irradiation and control group were decapitated. The rest were decapitated as follows: Irradiated group II 12 hours after the end of treatment, irradiated (III) and control (II) on the second day and irradiated (IV) and control (III) 7 days after treatment. There were only four animals in the second control group.

From all experimental animals biopsy specimens of skin were removed from (a) the irradiated field, (b) the gluteal region, (c) the left, fore-leg and also of (d) the trachea, (e) the oesophagus, (f) the forestomach, (g) the glandular stomach, (h) the small intestine (ileum), (i) the large intestine (colon), the outer orbital gland and the liver. The specimens were fixed in Bouin's solution and the sections stained with hemalaunecsin.

Counts of mitoses were made by calculating separately the mitotic figures per 2,000 cells according to a method previously described (6). The statistical significance of the results was tested by Student's t-test (2).

Another new experiment was performed in the same way as the first on rats of the same age. Similar x-ray therapy was administered to one group of 6 rats as above, but at 8 a.m. Six animals served as controls. Biopsy specimens were taken only 12 hours after the latest irradiation from the irradiated field, the gluteal region, the left fore-leg, the trachea, the oesophagus, the forestomach and the glandular stomach.

## RESULTS

The mitotic activity in the outer orbital gland and liver was the same both in the irradiated and control group at all the hours when the specimens were taken. The mitotic index was low (1—2) as usual. No were significant differences established in the specimens taken from the skin at the different times; the mitotic indexes were similar to those obtained in the earlier work. (7). The mitotic index of the forestomach and the glandular stomach after 12 hours was higher than in the control group, but there was no significant difference. No fluctuations were established in the mitotic index for the trachea, whereas the mitotic index for the oesophagus fell significantly in the irradiated group after 6 hours (Table 1).

The clearest differences in the mitotic index after irradiation were in the small intestine and large intestine. Compared with the controls, the counts in the first group after two days were nearly significantly higher in the irradiated animals and after seven days lower (Table 1). In the large intestine they were after  $\frac{1}{4}$  days significantly higher in the irradiated group after 6 hours and nearly significantly lower after 2 days (Table 1).

TABLE 1

| The Mitotic Index in Different Organs after Irradiation |                    |       |      |                    |       |      |        |       |      |        |       |      |
|---|--------------------|-------|------|--------------------|-------|------|--------|-------|------|--------|-------|------|
|   | $\frac{1}{4}$ Days |       |      | $\frac{1}{2}$ Days |       |      | 2 Days |       |      | 7 Days |       |      |
|   | Irr.               | Cont. | P <  | Irr.               | Cont. | P <  | Irr.   | Cont. | P <  | Irr.   | Cont. | P <  |
| Oesophagus ..   | 7.4                | 11.1  | 0.01 | 4                  | 1     | 0.05 |        |       |      |        |       |      |
| Small intestine   |                    |       |      |                    |       |      | 56.1   | 48    | 0.05 | 43.3   | 63.7  | 0.05 |
| Large intestine   | 37.3               | 22.8  | 0.01 |                    |       |      | 31.2   | 17.2  | 0.05 |        |       |      |

Half a day after irradiation the mitotic counts were higher than usual in the treated animals.

As there was no control group for this period a new experimental series was made in the same way as above from the following organs: oesophagus, trachea, the irradiated skin and the skin of the foreleg, forestomach and glandular stomach. Only the oesophageal mitotic count had risen almost significantly in the irradiation group (Table 1).

## DISCUSSION

Compared with the controls, statistically significant differences were established in the mitotic indexes in the irradiated groups only in the region of the alimentary canal. A significant drop in the mitotic index was established in the oesophagus of the irradiated group after 6 hours and an almost significant increase was demonstrated in the new experiment after 12 hours. The former must be regarded as a direct irradiation effect, since, according to the measurement made, the oesophagus received c. 30 per cent of the irradiation dose given to the skin, *i.e.* 900 r in all. The increased mitotic index in the new experiment, on the other hand, was caused by a compensatory increase following irradiation (4). Lower mitotic indexes after 12 hours, which is the usual finding, were due to the minimum phase of the diurnal rhythm (3).

The post-irradiation variations in the mitotic index in untreated areas of the skin were fairly small. Significance was established only in the labile organs which gave the highest mitotic index and in which cells regenerate rapidly. Variations were established most distinctly within 6 hours of irradiation, just as was seen previously with the mitotic indexes of the skin. An almost significant increase was still demonstrable after 2 days in the mitotic indexes of the large intestine of the irradiated animals. In the ileum, on the other hand, a compensatory decrease in the mitotic index was demonstrated after 7 days. This finding is attributed to the lability of the organ and its sensitive response to radiotherapy.

The absence in the present investigation of significant increases in the mitotic indexes of the skin of irradiated animals may be due to the fact that the cycle was different, as Chase *et al.* (1) have shown. The skin is not as susceptible to the distance effect at all cyclical phases. The low mitotic counts in the small and large intestine after x-irradiation are to be regarded as a kind of indirect and distance action of irradiation which can be transmitted humorally to molecules as a result of radiation energy. They cannot be regarded as a direct effect on the intestines since the environment of the therapeutic region was protected with lead and the scatter in the abdomen was only 5–8 mr with a dose of 1,000 r according to the measurements made.

## SUMMARY

1. The study comprised 52 rats, 3 months old, divided into first and second experiments. Twenty four rats divided into four groups were irradiated with short-distance irradiation, a daily dose of 1,000 r on three successive days. Sixteen rats in three groups served as controls. The area of irradiation was in the interscapular region of the skin. On termination of treatment biopsy specimens were removed after 6 and 12 hours, 2 and 7 days from the irradiated field, from two other regions of the skin and from different organs. In the second experiment biopsy specimens from the organs were taken only 12 hours after the last irradiation.

2. The clearest differences in the mitotic index were in the small intestine and large intestine after irradiation. The counts in the first group after 2 days were nearly significantly higher in the irradiated group and after seven days were lower than in the control group. In the large intestine they were significantly higher after 6 hours and after 2 days nearly significantly lower in the irradiated group. In the second experiment only in the oesophagus was the mitotic index nearly significantly higher. These phenomena can be attributed to humoral distance action.

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## LEUCINE AMINOPEPTIDASE IN THE RAT MAMMARY GLAND

A HISTOCHEMICAL STUDY

by

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(Received for publication July 12, 1961)

The mammary gland undergoes great physiological changes during puberty, pregnancy and lactation, and to some extent also in different phases of the estrous cycle. A number of investigators have stated that estrogen, progesterone and prolactin are involved in these changes (4, 9, 16). Prolactin sensitizes the mammae to sexual hormones, above all to estrogen (5). This hormone together with adrenocortical hormones is necessary for the beginning of lactation (8, 14). In fact, the normal function of the adrenal cortex and the thyroid gland is required for the growth and development of the mammae, although they exert no direct influence upon it (3, 18).

In addition to great morphological and cytological changes in different stages of development, changes occur also in enzymatic activity of the mammary tissue, especially toward the end of pregnancy and during lactation. Succinic dehydrogenase, arginase, cytochrome oxidase, hexokinase, glutamic dehydrogenase, for instance, increases in amount in the late part of pregnancy and during lactation expressing the intensity of oxidation and the protein synthesis in the mammary gland (2, 6, 7, 10, 11, 17).

A distinct increase occurs in leucine aminopeptidase activity of the serum during pregnancy (1). Though it has been known for a long time that the leucine aminopeptidase is associated with the protein synthesis, possible changes in leucine aminopeptidase



activity of the mammary gland during pregnancy, lactation or under the influence of hormones have not been investigated. In this paper the effects of various functional stages as well as the effects of hormones controlling the function of the mammary gland on «the histochemical leucine aminopeptidase» in this gland are, therefore, reported.

#### MATERIAL AND METHODS

Female albino rats were used as test animals. The mammary glands of virgin rats and of rats being in different stages of pregnancy or lactation period were compared with each other. Altogether 30 rats were used in these and 44 rats in experimental investigations.

The effect of various hormones was investigated by using the groups of 6 rats to which the following hormones were injected subcutaneously: «Dimenformon» Organon 1 mg daily for 5 days, «Progestin» Organon 5 mg daily for 5 days, «Sustanon» Organon 5 mg daily for 5 days, «Syntocinon» Sandoz 1 IU daily for 5 days. One group was oophorectomized and another adrenalectomized; both groups were killed 7 days after operation.

Eight lactating rats were weaned from the litters for two days. Some of them were then killed, and some again suckled for further two days after which they were also sacrificed.

After decapitation the mammary gland tissue was taken and sectioned with a cold knife microtome at 30–50  $\mu$ . Since the sections of the test animals had to be exactly as thick as those of the corresponding control group, the pieces of the mammary glands of the two types of animals were always sectioned in couples simultaneously and placed on the same slide.

Leucine aminopeptidase was demonstrated histochemically according to Nachlas, Crawford and Seligman (13). The possible effects of milk present in the lactating mammary glands was determined by adjusting either human, cow or rat milk into the incubation solutions of control slides. Preincubation of 30 minutes in milk was also proved. The effects of milk was studied also quantitatively in tissue homogenates by adding milk into the incubation medium used by Miller and Worsley (12). It was observed that milk contains some activity of leucine aminopeptidase but does not interfere the histochemical results.

## RESULTS

In the mammary gland from virgin and pregnant rats a strong activity of leucine aminopeptidase was observed. It was located in the walls of the glandular ducts and also in the epithelial buds representing the glandular tissue in the non-functioning gland (Fig. 1).

In the beginning of lactation the enzymatic activity fell sharply in the walls of the ducts; and it was minimal particularly in the alveolar epithelium (Fig. 2).

After two days weaning the enzymatic activity of the glandular epithelium increased markedly. If the animal began to suckle again, a fall, which was the same as that at the start of lactation, was observed in enzymatic activity.

Estrogen, progesterone, testosterone and oxytocin had no direct influence on the amount of enzyme in the glandular epithelium, although pronounced histological changes were present. No clear differences were found after oophorectomy and adrenalectomy.

## DISCUSSION

According to the present study a decrease was observed in the activity of leucine aminopeptidase after the beginning of lactation. At that time the protein synthesis increases in amount. An increase in the amount of enzymatic activity occurred after weaning, when the protein synthesis decreases in the glandular tissue. It seems likely, therefore, that leucine aminopeptidase activity does not fluctuate parallel to the protein synthesis of the mammary gland, rather the contrary. This is difficult to explain from the physiological point of view.

The role of leucine aminopeptidase in tissues is still obscure. It acts obviously hydrolytically converting amides and peptides into amino acids for intracellular use, and so it may be concerned with the synthesis of peptide bonds. Observations made by Pearce and Tremblay (15) indicate that leucine aminopeptidase may participate in the protein synthesis of the parathyroid glands. According to the present findings leucine aminopeptidase does not correlate to the casein synthesis in the mammary gland, for which reason it may be independent of leucine aminopeptidase.

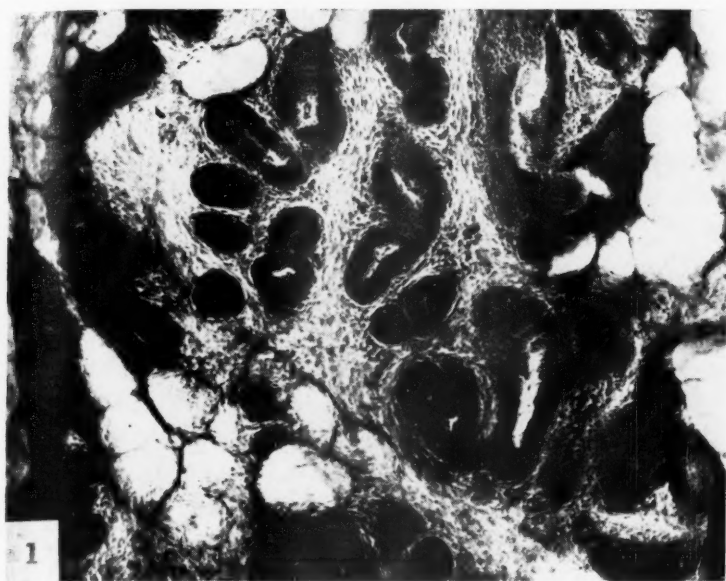


Fig. 1. Pregnant rat. A strong activity in the walls of the glandular ducts.  $\times 100$ .



Fig. 2. — Lactating rat. A slight activity in the mammary tissue.  $\times 50$ .

Greenbaum and Greenwood (7) studied the activities of glutamic dehydrogenase, glutamic-aspartic transaminase and cathepsin in the mammary gland. It has been suggested that these enzymes are associated with the protein synthesis of the mamma, while b-glucuronidase is considered as an example of an enzyme not playing a part in this synthesis during pregnancy, lactation or mammary involution after weaning. A parallelism has been shown between the activities of glutamic dehydrogenase and glutamic-aspartic transaminase and the lactational performance. The activity of b-glucuronidase and cathepsin increased sharply during early mammary involution. This fact is in agreement with our finding concerning leucine aminopeptidase. As for the role of cathepsin in the function of the mammary gland similar possibilities are presented as can be made concerning leucine aminopeptidase activity.

Our studies with hormone preparations show that pure preparations of estrogen, progesterone, testosterone or oxytocin alone, as well as oophorectomy and adrenalectomy as such one, does not have any effects marchant enough to be demonstrated by histochemical means on the leucine aminopeptidase, which suggests that it is controlled by some other probably more complicated mechanism.

#### SUMMARY

The histochemically demonstrable activity of leucine aminopeptidase in the mammary gland of virgin, pregnant and lactating rats was studied. The activity was very strong in the virgin and pregnant rat, and it was very slight in the lactating animal. The enzymatic activity was located in the walls of the glandular ducts and in the epithelium buds representing the glandular tissue in the non-functional gland. After weaning the activity increased rapidly. Estrogen, progesterone, testosterone and oxytocin had no effect. The physiological role of leucine aminopeptidase in the mammary gland is discussed.

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## THE EFFECT OF TEMPERATURE ON THE ELASTICITY OF FROG SKIN

by

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It is known that elastic tissue shows an increase in the elastic force with the temperature (4), whereas collagenous tissue shows a decrease. As these two components are found mixed in the skin (3), and as this tissue is in the frog found to give characteristical stress-strain curves (1), it is of interest to study the effect of temperature on these relations.

### METHOD

The method is fully described in an earlier paper (1). The principle of the method is based upon the stretching of a circular strip of the frog skin preparation with hydrostatic pressure. The recording of the pressure by means of an U-tube and a photocell and of the skin area by means of volume changes in the fluid system is made with a two-beam oscillograph (type Tektronix 502) on its two dimensional co-ordinate system.

The experiments were carried out with frog skin preparations (*Rana temporaria*) and 16 preparations were examined. Each skin strip (both dorsal and ventral skin) was first examined at a temperature of 21°C in the usual way by taking an area-pressure curve in which the stretch ranged to approx. 50 per cent of the in situ area. The temperature of the fluid in the cylinder on which the skin was fixed was then changed in the desired direction with a time gradient of approx. 1°C/min. The temperature was continuously followed

by means of an electrical thermometer, by fixing the measuring unit directly to the outside of the skin. At the same time, the temperature of the warmed fluid was measured. By this method, the measured temperature gradient on the two surfaces of the skin was  $< 1^{\circ}\text{C}$ . With each preparation, the temperature was changed only in one direction, after which the experiment was ended. Because of the phenomena of plasticity (2) in the stretching of frog skin, the area-pressure curve was estimated only once at every temperature examined. As a control experiment, the area-pressure curve of a rubber strip at a temperature of  $21^{\circ}\text{C}$  was determined.

## RESULTS

The Fig. 1 represents a skin at a temperature of  $21^{\circ}\text{C}$  and at a temperature of  $57^{\circ}\text{C}$ . In this figure the two recordings are corrected to real area-pressure curves (1). The horizontal axis gives the amount

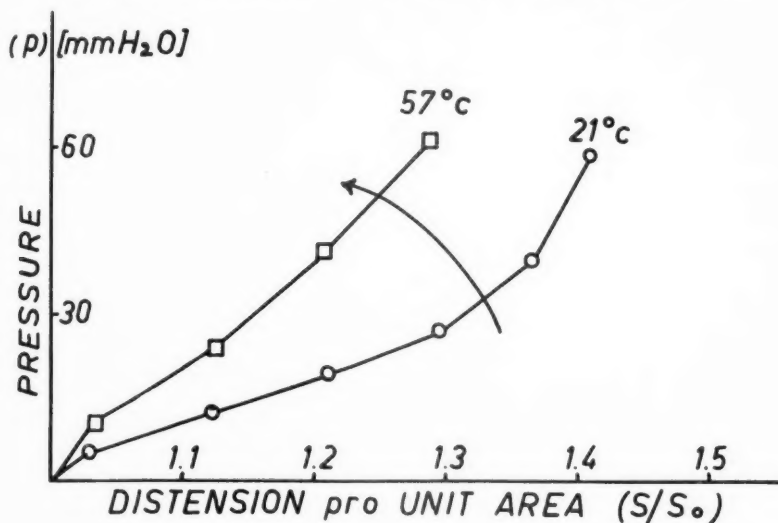


Fig. 1. — The effect of the increasing the temperature of the frog skin on the stress-strain relation of the skin.

of the stretch per unit area ( $S/S_0$ ). The vertical axis shows the pressure acting on the skin surface, in  $\text{mm H}_2\text{O}$ . It can be seen that the curve increases more rapidly at the higher temperature than at the lower.

In Fig. 2 are shown four curves, *i.e.* frog skin preparations at

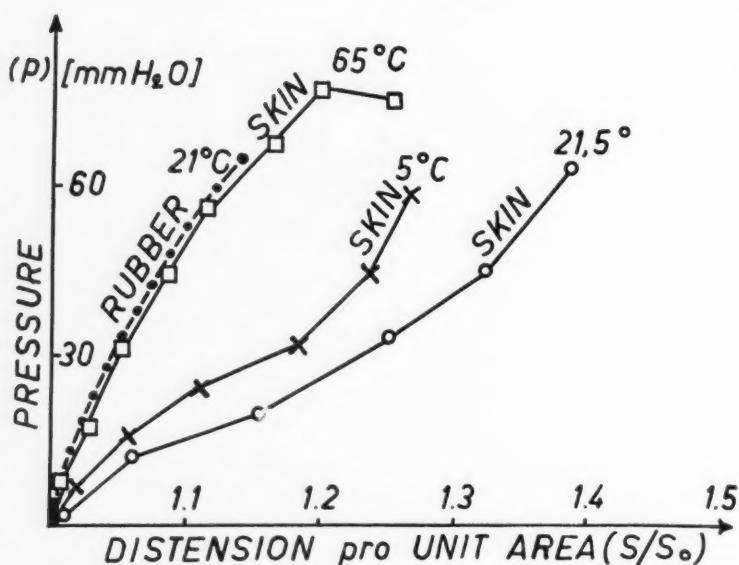


Fig. 2. — Area-pressure relations of frog skin preparations at temperatures of 5° C, 21.5° C and 65° C. The area-pressure curve of rubber at a temperature of 21° C is given. Note that the frog skin at 65° C is broken at a pressure of 80 mm H<sub>2</sub>O.

21.5°C, 5°C and 65°C, and a curve for rubber of the same thickness as the skin, at 21°C. The three skin-curves represent different skin preparations. It is seen that the effect of cooling changes the curve in the same direction as heating. However, the effect of cooling to 5°C is less pronounced. It is also seen that the curve of the skin at a temperature of 65°C resembles that of the rubber at a temperature of 21°C. The knick in the skin curve at the temperature of 65°C at a pressure of 80 mm H<sub>2</sub>O represents the point at which the skin was broken.

#### DISCUSSION

The effect of the elevation of the temperature on the elasticity of the skin was found to be an enhancement of the rubber-like behaviour of the skin. The stress-strain curves of the rubber are known to be of S-shape, if the stress is high enough. At low temperatures of the skin (about 5°C) the area-pressure relations were changed in the same direction as by high temperatures, but the effect was less pronounced. The result shows that shortening takes



place with elevation of temperature. This behaviour is analogous to that of many organic tissues, and is probably caused by the changes in proteins at temperatures where coagulation begins (3).

#### SUMMARY

The effect of temperature on the stress-strain relations of frog (*Rana temporaria*) skin preparations is studied. It is observed that the elasticity of the skin increases at low (below 5°C) and at high (above 21°C) temperatures. At 65°C the skin behaves in a rubber-like manner at low pressures acting on the skin.

This work is supported by a grant from the Finnish State Committee for Natural Sciences.

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## SOME OBSERVATIONS ON THE PLASTICITY OF FROG SKIN

by

RUDOLF M. BERGSTRÖM

(Received for publication May 18, 1961)

In an earlier study (1) concerning the elasticity of frog skin some phenomena were observed limiting the elastic behaviour of the skin tissue. According to Goodier and Hodge (2) the elastic behaviour of a material is characterized by the linear and reversible relationship between the stress acting on the material and the strain observed. The plasticity, again, is characterized by a non-linear and irreversible relation between the stress and strain. By reason of this phenomenon a creeping effect can be observed, which means that the material shows an increase in the size by constant load. On the other hand, a relaxation of the material can be observed, *i.e.* the material shows a decay of stress at constant size. In the study referred to above it could be observed that the stress-strain curves (determined as area-pressure curves according to the method used) were time-dependent. It was shown that the material lost its reversibility if stretched above a certain limit. On the other hand, it was observed that the shape of the curves was dependent on the speed at which the skin was loaded. The phenomenon of the plasticity is the object in the study to be described here.

### METHOD

The method used in the study was based on the same principle as in the earlier study concerning the elasticity of frog skin. Thus only a short description of this will be given here. The circular skin

strips, fresh prepared from normal healthy frogs (*Rana temporaria*), were placed on the end of a cylinder whose piston could be moved at a known speed by means of a motor. The cavity of the cylinder was connected with a U-tube and filled with frog's Ringer. By means of the U-tube and a photocell, the pressure acting on the skin surface and graduated by the position of the piston could be registered on the y-axis of a 2-dimensionally recording oscillograph (type Tektronix 502). The position of the piston, with a known relation to the area of the skin, could be registered (by means of a potentiometer) on the x-axis of the oscillograph. The registered curves were corrected to real area-pressure curves. In the earlier study, only statical area-pressure relations were examined, *i.e.* after every movement of the piston a considerable time interval elapsed before the recording was made.

In the experiments under consideration, two types of registration were made. One consisted of the same registration as described above (two-axis registration). The other was on moving film, with the oscillograph beams separately recording the time gradients of the photocell and the piston position voltage deflections. By means of this later method, the time gradients of the pressure acting on the skin surface and of the area of the skin could be determined.

About 20 skin preparations were used, and several experiments made with each of the skin strips. The temperature of the skin varied between 21° and 24° C during the experiments.

#### RESULTS

In Fig. 1 are given the corrected area-pressure curves for three experiments with different skin strips. The experiments were carried out at an intermediate speed of the piston displacement (1 ml/10 sec, which speed was satisfactorily followed by the apparatus), which corresponds to a change in the area of the skin of 0.4 cm<sup>2</sup>/sec.

Fig. 2 shows the result of unloading experiments, in which the preparations were stretched to a certain amount, and then left in this position for different periods of time. The representation is three-dimensional, the plane coordinates showing the degree of the stretch per area ( $S/S_0$ ) and the time ( $t$ ) from the beginning of the

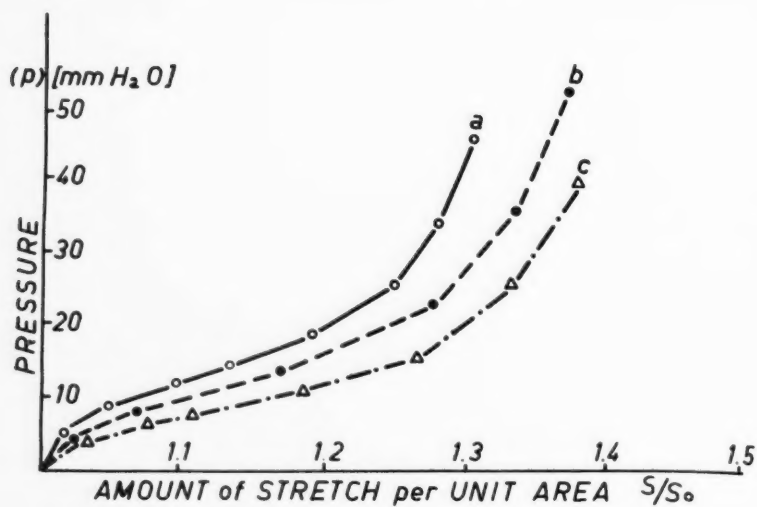


Fig. 1. — Three typical area-pressure curves of normal, fresh frog skins.

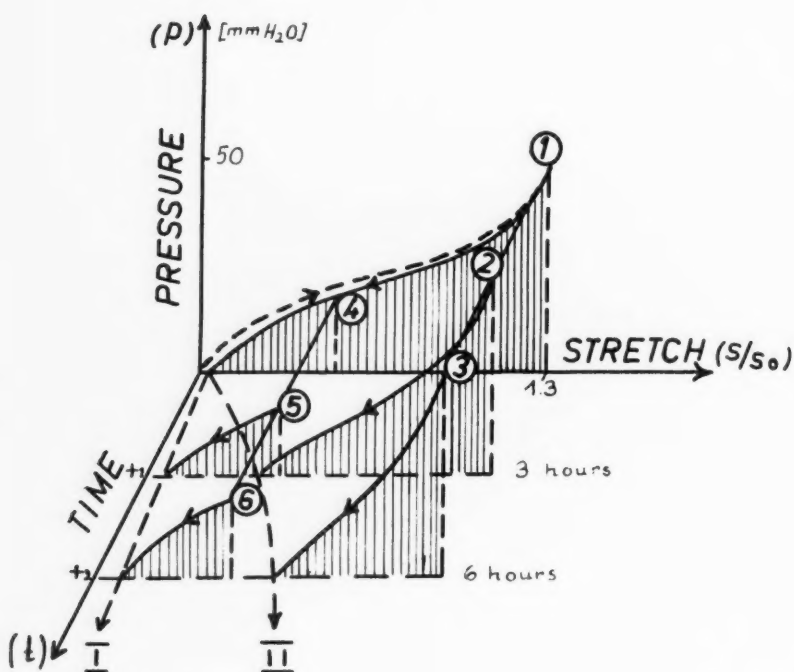


Fig. 2. — The time dependence of the area-pressure relation of frog skin (see text).

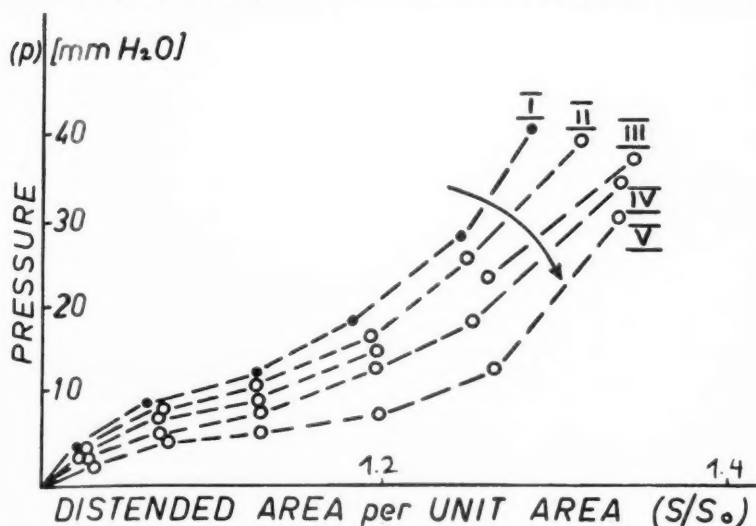


Fig. 3. — The effect of successive stretching on the area-pressure relation of frog skin.

experiment. The vertical dimension represents the pressure acting on the skin surface ( $p$ ). The dotted line on the  $(p, S/S_0)$ -plane shows the loading of the preparation, and curves 1—3 the unloadings from a relatively high degree of the stretch after the time interval indicated by the projections on the  $t$ -axis ( $t_1$  and  $t_2$ ). Curves 4—6 represent unloadings from a lower degree of stretch. It is seen that if left at a sufficiently degree of stretch, the skin does not re-assume its original area after the unloading (dotted lines I and II on the  $(t, S/S_0)$ -plane). The larger is the initial stretch, the greater is the deformation after the unloading and the longer time the stretch acts the greater the deformation. It was observed, however, that after a time of rest this deformation disappeared. This was the case only for fresh preparations, which recovered fully in a time of one to several hours, dependent on the initial stretch used. Older preparations (24 to 48 hours) did not, if stretched sufficiently, recover their natural state.

Fig. 3 shows the effect of the relaxation of the frog skin, if stretched several times within a short period of time. The curves of successive experiments (I—V) «fall» to the horizontal axis, i.e. the skin is distended to a greater area with a certain pressure in the latter distensions.

## DISCUSSION

As observed in an earlier study on the elasticity of the frog skin (1), the frog skin shows reversible and linear behaviour if stretched to a maximum of one tenth of its area. The reversible but non-linear behaviour ranges to a stretch of approx. two tenths of the initial area after which the stretch effect is irreversible. The behaviour is termed plastic (2) in the two latter cases, *i.e.* after the first tenth of the stretch, when the behaviour is nonlinear (reversible or irreversible). The two most remarkable phenomena of plasticity, the relaxation and the creep (3) are observed. Thus it is concluded that frog skin is highly sensitive to long-lasting distensions.

## SUMMARY

The plasticity of frog (*Rana temporaria*) skin preparations was studied, attention being paid to the two most remarkable phenomena of plasticity, the relaxation and the creep. It was concluded that frog skin is highly sensitive to long-lasting distensions.

This work was aided by a grant from The Finnish State Committee for Natural Sciences.

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## STUDIES IN GLUTATHIONE REDUCTASE OF THE LENS

by

AIMO KÄRKELÄ and PENTTI MIETTINEN

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The present writers have previously studied the changes in the activity of glutathione reductase (GR) with the maturation of senile cataract and demonstrated the decrease that occurs in activity (5).

When the cataract matures, the SH-group is oxidised into the S—S form. It has been shown that GSH decreases distinctly in the lens during the development of cataract, while the oxidised form increases (2). GR is capable in tissue of converting GSSG to GSH. Reduced triphosphopyridine nucleotide (cozymase II) is necessary for the reaction.

### METHODS

The writers measured the activity of GR from extract of lenses with various degrees of opacification.

Micromodifications of the methods introduced by van Heyningen and Pirie (4) and by Racker (1) were employed in the determinations. Incubated at 26°C in phosphate buffer, pH 7.25. Liberated GSH was determined by the nitroprusside reaction (3).

The activity of GR was expressed in micromols of the GSH liberated in 10 min. in the test conditions from the substrate by the GR present in one gram of lens protein.

GRAPH 1  
THE pH CURVE OF GLUTATHIONE REDUCTASE

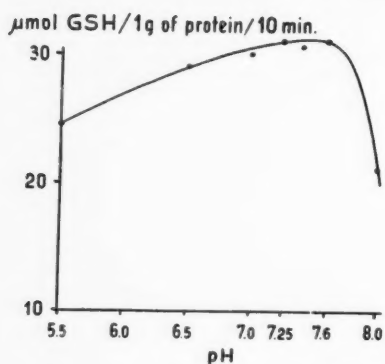


Fig. 1.

GRAPH 2  
RATIO BETWEEN THE QUANTITY OF GSH LIBERATED  
(FORMATION OF COLOUR) AND INCUBATION TIME

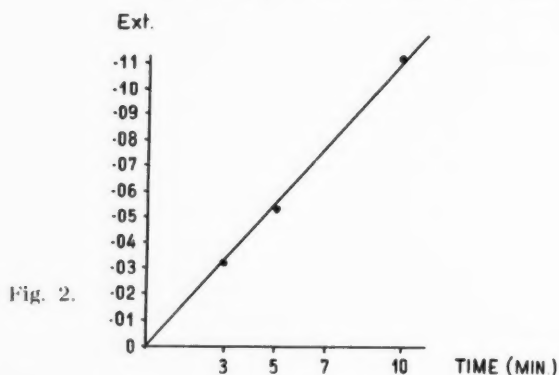


Fig. 2.

GRAPH 3.  
RATIO BETWEEN THE ACTIVITY OF GR (FORMATION  
OF COLOUR DUE TO GSH LIBERATED IN 10 MIN.)  
AND THE AMOUNT OF LENS HOMOGENATE.

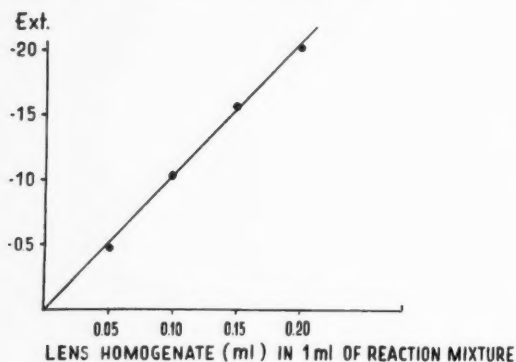


Fig. 3.



## RESULTS

The results are given in the table and graphs 1, 2, and 3.

TABLE  
ACTIVITY OF GR IN LENSES ( $\mu$ MOL/1 G OF PROTEIN/10 MIN.)

|      | Incipient and<br>Feremature | Mature         | Hyperature     |
|------|-----------------------------|----------------|----------------|
|      | GR act<br>11—28             | GR act<br>3—20 | GR act<br>2—15 |
| Mean | 20.8                        | 10.5           | 8.2            |
|      | $\pm$ 5.6                   | $\pm$ 5.2      | $\pm$ 4.3      |

The average activity of GR in cases of incipient and feremature cataract is 2—2.5 times greater than in the mature forms.

## SUMMARY

The effect of pH, incubtion period aud enzyme concentration on GR activity is illustrated and the decrease in activity with the maturation of cataract is shown in tabular form.

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## THE ACTION OF ETHYLENE OXIDE ON POLIO VIRUS ADSORBED ON HELA CELLS

REMOVAL OF GAS AFTER TREATMENT OF CELL CULTURES WITH  
ETHYLENE OXIDE

by

VELJO RAUNIO and ANJA TAIPALE

(Received for publication July 11, 1961)

Ethylene oxide not only possesses viricidal properties but also exerts a toxic effect on tissues growing in vitro. When viruses exposed to ethylene oxide are added to tissue, degenerative changes induced by ethylene oxide are observed first. Such changes are observed even when the virus suspension has been diluted 10000-fold. If this non-specific effect of the ethylene oxide were weaker, the viricidal power of the gas could be determined quantitatively by titration (1). An attempt was therefore made to develop a method by which the ethylene oxide could be rapidly removed from the treated virus sample before adding the latter to the tissue culture. A method for this purpose has been described previously in which the gas has been removed by centrifugation in vacuo (2). However, the infectivity of viruses has been found to decrease when this method has been used and the method cannot hence be considered quite satisfactory. In the new method the viruses are first added to the tissue cells, the cells are then exposed to the gas and the gas is finally removed by washing the cells.

### METHOD

*The Adsorption of Viruses to HeLa Cells.* — The present method thus differs from earlier ones in that the gas is allowed to interact with the viruses after these have become adsorbed on the cells.

The virus suspensions (polio virus type 1, Brunhilde, I.D. $10^{-6.5}$ ) employed contained a large number of virus particles so that every tissue cell became infected simultaneously. The virus suspension (2 ml) was added to HeLa monolayer cell cultures containing an average of 50,000 cells. The time allowed for the adsorption was 4 hours. At the end of this period about one thousandth of the original infectivity was retained in the cells and the viruses had not yet begun to multiply (3). The growth medium was removed from the tissue culture tubes before the infected tissue cells were treated with the gas.

*Treatment with Gas.* — In our earlier studies we employed a mixture of ethylene oxide and carbon dioxide, but we have recently diluted the ethylene oxide with a gaseous chlorinated and fluorinated hydrocarbon (dichlorodifluoromethane, commercially sold under the names Freon and Frigen.) The mixture of ethylene oxide and dichlorodifluoromethane was supplied in metal cans equipped with a valve. Owing to the small size and light weight of the cans and the simple valve construction, the containers are much more convenient to handle than large pressure vessels. It was not possible to obtain the gas mixture at a pressure exceeding 0.8 atmosphere because the gas sterilisation unit available did not work at higher pressures (gas chamber 4—43, Oy Santasalo-Sohlberg Ab). Since preliminary tests revealed that the inactivation of the viruses could be effected relatively rapidly at this pressure when the temperature was 20°C, higher temperatures were not employed. The time the gas acted upon the infected tissue cells was 10—40 minutes.

*Removal of Gas after Exposure.* — If the gas-treated cells are transferred directly to HeLa cell cultures, the cells in the latter are destroyed rapidly. When, however, the exposed cells were washed twice with Hanks salt solution, no degenerative changes due to the gas were observed in subcultures. The cells are readily detached from the walls of the test tubes after the exposure to the gas and can be readily dispersed in the mentioned solution. The washing of the cells is performed in the usual manner by centrifugation at a low speed.

*Titration.* — The determination of the I.D.<sub>50</sub> was performed in the usual manner (Reed and Muench). The infected cells from

two test tubes were taken to prepare the first dilution. The final readings were recorded after an incubation period of five days, but further subcultures were made when found necessary.

#### RESULTS AND DISCUSSION

As already mentioned, only one thousandth of the infectivity of the viruses was retained by the HeLa cells after an adsorption time of four hours. This was due, first, to the small number of cells relative to the number of viruses and, second, to the fact that the viruses were in the eclipse phase (7).

The decrease in the infectivity with time is shown in the Figure and Table. The values plotted are the averages of four values from closely comparable experiments. The standard error of the means were computed according to Bingel (6). The standard errors were not significant. The Brunhilde polio virus is inactivated much more rapidly than other viruses studied previously (2). The infected cell suspensions which were exposed to the gas for 40 minutes did not exhibit any virus activity even when they were subcultured several times. In order to confirm that the dichlorodifluoromethane was not solely responsible for the decrease in infectivity, control

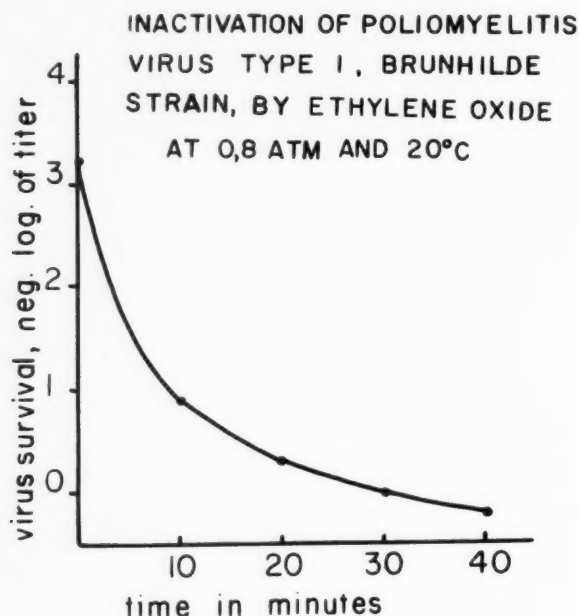


TABLE 1

EFFECT OF ETHYLENE OXIDE ON POLIOVIRUS TYPE 1 (BRUNHILDE). VIRUS SURVIVAL EXPRESSED AS NEG LOG OF TITER (TCID<sub>50</sub>)

| Duration of Gas Treatment at +20 C and 0.8 atm.  | The Average of the Values from 4 Closely Comparable Experiments |
|--|---|
| 0 min  | 3.23 $\pm$ 0.17   |
| 10 min.  | 0.88 $\pm$ 0.15   |
| 20 min.  | 0.31 $\pm$ 0.03   |
| 30 min.  | 0.0   |
| 40 min.  | no survival   |
| Controls   |   |
| 1. Uninfected cells treated with gas, washed twice and subcultured on HeLa monolayer cellcultures (wash control) | no changes in subcultures                                       |
| 2. normal cells  | no changes  |
| 3. Infected cells treated 40 min. with freon only and subcultured on HeLa cell cultures (Freon control)          | no loss of infectivity  |

experiments were also carried out using dichlordifluoromethane alone. The gas was readily eliminated by the described washing procedure. The elimination of the gas by Hanks solution is evidently due to the neutralising action of the salts in the solution (5) and to the diffusion of the gas molecules into the solution.

## SUMMARY

It has been found that the polio virus type 1 Brunhilde adsorbed on HeLa cells loses its infectivity when it is exposed to ethylene oxide (ethylene oxide and dichlordifluoromethane at 0.8 atmosphere and 20C°) for a period of 40 minutes.

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## EXCRETION OF ANDROGENS, ESPECIALLY DEHYDROEPIANDROSTERONE, IN HYPEREMESIS GRAVIDARUM

by

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(Received for publication July 13, 1961)

In recent years numerous investigations have been published on the significance of the adrenal cortex in hyperemesis gravidarum (1, 4, 6, 8, 9, 10, 11, 12, 13, 14, 15, 16). During the first months of gestation the adrenocortical function is normal or slightly below normal (1, 11). Actual adrenocortical insufficiency has been observed in patients with hyperemesis gravidarum (1, 4, 6, 8, 9, 10, 11, 12, 13, 17).

In an earlier investigation Järvinen and Uuspää (8, 9) studied the excretion of 11-oxycorticosteroids and total 17-ketosteroids (17-KS) in hyperemesis gravidarum. To obtain further information on the adrenocortical function of these patients we determined in the present study the total 17-KS excretion and that of dehydroepiandrosterone (D).

### MATERIAL AND METHODS

To study the effect of corticotrophin stimulation on the adrenocortical function 42 patients with hyperemesis were treated by administering 20 IU of corticotrophin per day on 5 successive days. The same treatment received also 5 non-vomiting patients in the first trimester of pregnancy.

Collection of 24-hours urine for hormone determinations was done before commencement of this treatment, on the first and second days of treatment, on the day following discontinuation of corticotrophin administration, and a few days later on discharge of the patient from the hospital. The total 17-KS were determined by the method C of Jensen and Tötterman (7) and the dehydroepiandrosterone (D) by the technique of Jensen (3). D was extracted from 1/100th part of the 24 hours urine volume and the standard solution used in the determinations contained 50  $\mu$ g of this steroid. If the reading of the analysed solution was lower than that of the standard solution, the final result is stated as  $< 50 \mu$ g. It is a well known fact that in isolation of 17-KS (during hydrolysis) D does not tolerate high temperatures or the action of strong mineral acids. Both environments cause the destruction of a part of D during hydrolysis for the determination of total 17-KS. If the D content of urine is high, as a result of destruction, the amount of total 17-KS can be smaller than the amount of D according to Jensen.

The differences between the values of 17-KS and D of different groups of patients as well as the differences between the values before and after 5 days corticotrophin treatment in each group are tested statistically by applying the t-test.

#### RESULTS

In the control group of non-vomiting pregnant patients (table 1) were the values of total 17-KS slightly lower than the corresponding values of nonpregnant women in the same age (5) but the difference was not significant. The absolute values of D on their part were significantly lower than the corresponding values of non-pregnant women (18) and the difference between the percentile values of D was highly significant (table 2). After 5 days corticotrophin administration increased the excretion of both the total 17-KS and D highly significantly in this group.

The total 17-KS excretion was on the same level on pregnant women in the first trimester without or with hyperemesis (table 2). The results obtained in the present study of patients with hyperemesis are divided into three groups according to the percentage of D. Group I includes the cases in which the proportion of D was

TABLE 1  
EXCRETION OF 17-KETOSTEROIDS (17-KS) AND DEHYDROEPIANDROSTERONE (D)  
BEFORE AND AFTER CORTICOTROPHIN ADMINISTRATION IN THE CONTROL GROUP  
(IN MG/24 HRS)

| No/Age | Parity   | Day of<br>Pregnancy | Total<br>Amount<br>of Cortico-<br>trophin | Day of<br>Adminstr.<br>and<br>Controls* | 17-KS | D (%)        |
|--------|----------|---------------------|---|---|-------|--------------|
| 50/35  | III      | 78                  | 100 IU                                    | C                                       | 4.2   | 0.2 (4.8)    |
|        |          |                     |   | 1                                       | 6.8   | 0.4 (5.9)    |
|        |          |                     |   | 2                                       | 7.8   | 0.2 (2.6)    |
|        |          |                     |   | 6                                       | 9.0   | 1.2 (13.3)   |
|        |          |                     |   | C                                       | 5.8   | 0.4 (6.9)    |
| 51/36  | VIII (X) | 88                  | 100 IU                                    | C                                       | 5.4   | 0.2 (3.7)    |
|        |          |                     |   | 1                                       | 6.8   | 0.6 (8.8)    |
|        |          |                     |   | 2                                       | 6.6   | 0.5 (7.6)    |
|        |          |                     |   | 6                                       | 9.8   | 0.9 (9.2)    |
|        |          |                     |   | C                                       | 5.5   | 0.2 (3.6)    |
| 52/31  | IV       | 91                  | 100 IU                                    | C                                       | 7.3   | 0.5 (6.9)    |
|        |          |                     |   | 1                                       | 7.1   | 0.5 (7.1)    |
|        |          |                     |   | 2                                       | 8.8   | 0.4 (4.6)    |
|        |          |                     |   | 6                                       | 8.9   | 0.4 (4.5)    |
|        |          |                     |   | C                                       | 6.4   | 0.2 (3.1)    |
| 53/34  | VI (IX)  | 88                  | 100 IU                                    | C                                       | 12.5  | 0.6 (4.8)    |
|        |          |                     |   | 1                                       | 12.2  | 0.9 (7.4)    |
|        |          |                     |   | 2                                       | 12.7  | 0.7 (5.5)    |
|        |          |                     |   | 6                                       | 14.1  | 0.9 (6.4)    |
|        |          |                     |   | C                                       | 6.6   | 0.3 (4.6)    |
| 54/23  | III      | 103                 | 100 IU                                    | C                                       | 3.8   | < 50 $\mu$ g |
|        |          |                     |   | 1                                       | 5.7   | 1.8 (31.6)   |
|        |          |                     |   | 2                                       | 6.6   | 1.3 (19.7)   |
|        |          |                     |   | 6                                       | 6.4   | 1.0 (15.6)   |
|        |          |                     |   | C                                       | 3.7   | 0.5 (13.5)   |

\* C = control before and after corticotrophin administration 1, 2, 6 = 1, 2., 6. day of treatment with corticotrophin

TABLE 2  
THE MEAN EXCRETION (IN MG/24 HRS) OF 17-KS AND D BEFORE AND AFTER  
5 DAYS TREATMENT WITH CORTICOTROPHIN AS WELL AS THE CORRESPONDING  
MEAN EXCRETION OF NON-PREGNANT WOMEN AT THE SAME AGE (5, 18)

|                   | No. of<br>Cases | Age  | Before the<br>Treatment |            | After<br>Treatment |            | Mean Values for<br>Non-pregnant<br>Women |            |
|-------------------|-----------------|------|-------------------------|------------|--------------------|------------|--|------------|
|                   |                 |      | 17-KS                   | D (%)      | 17-KS              | D (%)      | 17-KS                                    | D (%)      |
| Control-<br>group | 5               | 32   | 6.6                     | 0.3 (4.5)  | 9.6                | 0.9 (9.5)  | 7.2                                      | 1.4 (18.3) |
| Group I           | 14              | 30   | 5.7                     | 0.2 (3.5)  | 9.0                | 0.8 (8.9)  | 7.4                                      | 1.3 (17.3) |
| Group II          | 14              | 26.5 | 6.5                     | 0.9 (13.8) | 13.7               | 2.7 (19.7) | 8.6                                      | 1.2 (13.9) |
| Group III         | 14              | 27.5 | 8.8                     | 3.0 (34.1) | 12.2               | 6.3 (51.6) | 8.2                                      | 1.3 (15.4) |



TABLE 3

EXCRETION OF 17-KS AND D (IN MG/24 HRS) OF PATIENTS WITH HYPEREMESIS IN GROUP I

| No/Age | Parity | Day of Pregnancy | Total Amount of Corticotrophin | Day of Administr. and Controls | 17-KS | D (%)        |
|--------|--------|------------------|--------------------------------|--------------------------------|-------|--------------|
| 3/37   | IV     | 81               | 100 IU                         | C                              | 5.3   | 0.3 (5.7)    |
|        |        |                  |                                | 1                              | 7.5   | 0.6 (8.0)    |
|        |        |                  |                                | 2                              | 6.7   | 0.3 (4.5)    |
|        |        |                  |                                | 6                              | 7.5   | 0.3 (4.0)    |
| 4/40   | VII    | 40               | 100 IU                         | C                              | 8.0   | 0.4 (5.0)    |
|        |        |                  |                                | 1                              | 5.3   | 0.3 (5.7)    |
|        |        |                  |                                | 2                              | 10.6  | 0.8 (7.5)    |
|        |        |                  |                                | 6                              | 11.8  |              |
| 6/30   | III    | 60               | 100 IU                         | C                              | 7.0   | 0.7 (10.0)   |
|        |        |                  |                                | C                              | 6.7   | 0.5 (7.5)    |
|        |        |                  |                                | 1                              | 7.6   | 1.8 (23.7)   |
|        |        |                  |                                | 2                              | 6.2   | 0.8 (12.9)   |
| 9/27   | I      | 69               | 100 IU                         | 6                              | 12.8  | 3.9 (30.5)   |
|        |        |                  |                                | C                              | 6.1   | 0.3 (4.9)    |
|        |        |                  |                                | C                              | 13.9  |              |
|        |        |                  |                                | 1                              | 8.1   | 0.4 (4.9)    |
| 17/33  | III    | 48               | 200 IU                         | 2                              | 10.3  | 0.6 (5.8)    |
|        |        |                  |                                | 6                              | 13.5  | 0.7 (5.2)    |
|        |        |                  |                                | C                              | 10.0  | 0.5 (5.0)    |
|        |        |                  |                                | C                              | 4.7   | < 50 $\mu$ g |
| 18/26  | III    | 72               | 140 IU                         | 1                              | 6.4   | 0.5 (7.8)    |
|        |        |                  |                                | 2                              | 7.9   | 0.6 (7.6)    |
|        |        |                  |                                | 6                              | 9.9   | 0.9 (9.1)    |
|        |        |                  |                                | C                              | 9.0   | 0.6 (6.7)    |
| 22/31  | V      | 125              | 140 IU                         | C                              | 7.7   | 0.7 (9.1)    |
|        |        |                  |                                | 1                              | 8.0   | 0.5 (6.3)    |
|        |        |                  |                                | 2                              | 13.3  | 0.6 (4.5)    |
|        |        |                  |                                | 6                              | 17.1  | 1.3 (7.6)    |
| 23/32  | IV     | 72               | 140 IU                         | C                              | 18.4  | 1.8 (9.8)    |
|        |        |                  |                                | C                              | 4.8   | < 50 $\mu$ g |
|        |        |                  |                                | 1                              | 5.1   | < 50 $\mu$ g |
|        |        |                  |                                | 2                              | 5.5   | < 50 $\mu$ g |
|        |        |                  |                                | 6                              | 6.2   | < 50 $\mu$ g |
|        |        |                  |                                | C                              | 3.1   | 0.2 (6.5)    |
|        |        |                  |                                | 1                              | 4.8   | 0.5 (10.4)   |
|        |        |                  |                                | 2                              | 6.0   | 0.7 (11.7)   |
|        |        |                  |                                | 6                              | 6.8   | 0.5 (7.4)    |
|        |        |                  |                                | C                              | 6.3   | 0.2 (3.2)    |

TABLE 3 (Continued).

| No/Age | Parity | Day of Pregnancy | Total Amount of Corticotrophin | Day of Administr. and Controls | 17-KS | D (%)        |
|--------|--------|------------------|--------------------------------|--------------------------------|-------|--------------|
| 24/30  | III    | 53               | 100 IU                         | C                              | 4.2   | 0.3 (7.1)    |
|        |        |                  |                                | 1                              | 4.1   | < 50 $\mu$ g |
|        |        |                  |                                | 2                              | 3.0   | < 50 $\mu$ g |
|        |        |                  |                                | 6                              | 5.7   | < 50 $\mu$ g |
|        |        |                  |                                | C                              | 6.3   | 1.3 (20.6)   |
| 32/26  | II     | 57               | 100 IU                         | C                              | 4.4   | 0.1 (2.3)    |
|        |        |                  |                                | 1                              | 8.4   | 1.4 (16.7)   |
|        |        |                  |                                | 6                              | 13.3  | 1.5 (11.3)   |
|        |        |                  |                                | C                              | 4.6   | < 50 $\mu$ g |
| 30/25  | II     | 58               | 100 IU                         | C                              | 4.8   | < 50 $\mu$ g |
|        |        |                  |                                | 1                              | 2.8   | 0.2 (7.1)    |
|        |        |                  |                                | 2                              | 3.1   | < 50 $\mu$ g |
|        |        |                  |                                | C                              | 2.7   | < 50 $\mu$ g |
| 33/19  | I      | 100              | 100 IU                         | C                              | 4.5   | 0.1 (2.2)    |
|        |        |                  |                                | 1                              | 9.6   | 1.2 (12.5)   |
|        |        |                  |                                | 2                              | 8.5   | 1.4 (16.5)   |
|        |        |                  |                                | 6                              | 3.5   | < 50 $\mu$ g |
|        |        |                  |                                | C                              | 4.9   | 0.2 (4.1)    |
| 46/19  | I      | 83               | 100 IU                         | C                              | 4.2   | < 50 $\mu$ g |
|        |        |                  |                                | 1                              | 3.5   | 0.5 (14.3)   |
|        |        |                  |                                | 2                              | 5.7   | 1.0 (17.5)   |
|        |        |                  |                                | 6                              | 2.7   | 0.2 (7.4)    |
|        |        |                  |                                | C                              | 5.1   | 0.3 (5.9)    |
| 47/35  | VI     | 84               | 100 IU                         | C                              | 2.9   | < 50 $\mu$ g |
|        |        |                  |                                | 1                              | 3.5   | 1.5 (42.9)   |
|        |        |                  |                                | 2                              | 10.3  | 1.9 (18.4)   |
|        |        |                  |                                | 6                              | 6.1   | < 50 $\mu$ g |
|        |        |                  |                                | C                              | 6.0   | 0.1 (1.7)    |

1—9.9 per cent of total 17-KS, group II the cases with D in the range 10—19.9 per cent and group III those in which D was  $\geq$  20 per cent (tables 3, 4, 5).

*Group I.* — The amounts of 17-KS and D were in this group quite on the same level as in the control group. After 5 days corticotrophin treatment increased the amount of total 17-KS highly significantly also in this group but the amounts of D showed only slight changes that were not significant.

TABLE 4  
EXCRETION OF 17-KS AND D (IN MG/24 HRS) OF PATIENTS WITH HYPEREMESIS  
IN GROUP II

| No/Age | Parity | Day of<br>Pregnancy | Total<br>Amount<br>of Cortico-<br>trophin | Day of<br>Adminstr.<br>and<br>Controls | 17-KS | D (%)        |
|--------|--------|---------------------|---|--|-------|--------------|
| 5/30   | II     | 45                  | 100 IU                                    | C                                      | 6.6   | 0.8 (12.1)   |
|        |        |                     |   | 2                                      | 6.4   |              |
|        |        |                     |   | C                                      | 8.6   |              |
| 8/25   | III    | 89                  | 100 IU                                    | C                                      | 5.5   | < 50 $\mu$ g |
|        |        |                     |   | 1                                      | 7.0   |              |
|        |        |                     |   | 2                                      | 9.8   | 0.7 (12.7)   |
|        |        |                     |   | 6                                      | 17.6  |              |
|        |        |                     |   | C                                      | 9.0   |              |
| 14/18  | I      | 73                  | 100 IU                                    | C                                      | 6.2   | 0.6 (9.7)    |
|        |        |                     |   | 1                                      | 3.8   |              |
|        |        |                     |   | 6                                      | 12.0  | < 50 $\mu$ g |
|        |        |                     |   | C                                      | 8.2   |              |
| 15/23  | I      | 100                 | 100 IU                                    | C                                      | 5.0   | 0.8 (16.0)   |
|        |        |                     |   | 1                                      | 5.0   |              |
|        |        |                     |   | 2                                      | 7.9   | < 50 $\mu$ g |
|        |        |                     |   | 6                                      | 7.1   |              |
|        |        |                     |   | C                                      | 7.1   |              |
| 16/31  | IV     | 145                 | 140 IU                                    | C                                      | 4.1   | 0.5 (12.2)   |
|        |        |                     |   | 1                                      | 4.9   |              |
|        |        |                     |   | 6                                      | 9.4   | 1.0 (20.1)   |
|        |        |                     |   | C                                      | 4.4   |              |
| 19/32  | I      | 87                  | 110 IU                                    | C                                      | 8.6   | 0.4 (5.6)    |
|        |        |                     |   | 1                                      | 10.3  |              |
|        |        |                     |   | 2                                      | 9.5   | 1.0 (11.6)   |
|        |        |                     |   | 6                                      | 9.5   |              |
|        |        |                     |   | C                                      | 4.4   | 2.1 (20.4)   |
|        |        |                     |   | C                                      | 12.3  |              |
| 20/29  | III    | 45                  | 100 IU                                    | 1                                      | 14.1  | 0.9 (9.1)    |
|        |        |                     |   | 2                                      | 13.6  |              |
|        |        |                     |   | 6                                      | 25.0  | 1.5 (15.8)   |
|        |        |                     |   | C                                      | 13.0  |              |
|        |        |                     |   | C                                      | 6.3   | 2.0 (16.3)   |
| 26/32  | I      | 65                  | 100 IU                                    | 1                                      | 12.2  |              |
|        |        |                     |   | 6                                      | 15.1  | 4.5 (31.9)   |
|        |        |                     |   | C                                      | 13.2  |              |
|        |        |                     |   | C                                      | 7.6   | 3.8 (27.9)   |
| 28/21  | I      | 57                  | 100 IU                                    | 1                                      | 10.6  |              |
|        |        |                     |   | 2                                      | 7.7   | 9.0 (36.0)   |
|        |        |                     |   | C                                      | 8.2   |              |
|        |        |                     |   | C                                      | 8.2   | 1.7 (13.1)   |
|        |        |                     |   | C                                      | 6.3   |              |
|        |        |                     |   | C                                      | 6.3   | 1.5 (9.9)    |
|        |        |                     |   | C                                      | 13.2  |              |
|        |        |                     |   | C                                      | 7.6   | 1.3 (9.8)    |
|        |        |                     |   | C                                      | 7.6   |              |
|        |        |                     |   | C                                      | 7.6   | 8.0 (75.5)   |
|        |        |                     |   | C                                      | 7.6   |              |
|        |        |                     |   | C                                      | 7.6   | 11.0 (142.8) |
|        |        |                     |   | C                                      | 7.6   |              |

TABLE 4 (Continued).

| No/Age | Parity | Day of Pregnancy | Total Amount of Corticotrophin | Day of Administr. and Controls | 17-KS | D (‰)      |
|--------|--------|------------------|--------------------------------|--------------------------------|-------|------------|
| 34/25  | I      | 60               | 100 IU                         | C                              | 3.7   | 0.7 (18.9) |
|        |        |                  |                                | 1                              | 8.6   | 1.7 (19.8) |
|        |        |                  |                                | 2                              | 6.0   | 0.4 (6.7)  |
|        |        |                  |                                | C                              | 6.6   | 0.1 (1.5)  |
| 35/32  | III    | 63               | 100 IU                         | C                              | 6.5   | 1.0 (15.4) |
|        |        |                  |                                | 2                              | 12.4  | 3.6 (29.0) |
|        |        |                  |                                | C                              | 4.2   | < 50 µg    |
| 39/25  | III    | 77               | 120 IU                         | C                              | 3.6   | 0.5 (13.9) |
|        |        |                  |                                | 1                              | 4.2   | 0.3 (7.1)  |
|        |        |                  |                                | 2                              | 4.8   | 0.6 (12.5) |
|        |        |                  |                                | C                              | 5.1   | 0.8 (15.7) |
| 43/25  | III    | 99               | 120 IU                         | C                              | 8.0   | 1.0 (12.5) |
|        |        |                  |                                | 1                              | 8.8   | 2.1 (23.9) |
|        |        |                  |                                | 2                              | 8.0   | 2.3 (28.8) |
|        |        |                  |                                | C                              | 6.8   | 0.4 (5.9)  |
| 44/22  | I      | 72               | 120 IU                         | C                              | 7.1   | 1.4 (19.7) |
|        |        |                  |                                | 1                              | 9.4   | 3.7 (39.4) |
|        |        |                  |                                | 2                              | 11.6  | 3.6 (31.0) |
|        |        |                  |                                | C                              | 9.0   | 1.4 (15.6) |

*Group II.* — Also in this group were the values of total 17-KS on the same level as in the control group and in nonpregnant women of the same age. The absolute and percentile values of D were also on the same level as those of nonpregnant women, that is the absolute values of D were significantly and the percentile values highly significantly higher than in our control group and in group I. After corticotrophin administration increased the values of total 17-KS highly significantly also in this group. The values of D showed only a slight rise that was not statistically significant.

*Group III.* — The excretion of 17-KS was in this group slightly higher than in the former groups and in the control group but the difference was not significant. Both the absolute and percentile values of D were in this group highly significantly higher than in the control group and in the other groups of hyperemesis as well as in nonpregnant women of the same age. The difference between the values of 17-KS and D before and after corticotrophin treatment was not statistically significant in this group.

TABLE 5

EXCRETION OF 17-KS AND D OF PATIENTS WITH HYPEREMESIS IN GROUP III  
(IN MG/24 HRS)

| No/Age | Parity | Day of Pregnancy | Total Amount of Corticotrophin | Day of Agministr. and Controls | 17 KS | D (%)        |
|--------|--------|------------------|--------------------------------|--------------------------------|-------|--------------|
| 1/21   | I      | 62               | 80 IU                          | C                              | 12.7  | 7.2 (56.7)   |
|        |        |                  |                                | 1                              | 10.1  | 7.5 (74.3)   |
|        |        |                  |                                | 2                              | 11.7  | 7.8 (66.7)   |
|        |        |                  |                                | 6                              | 12.0  | 4.8 (40.0)   |
|        |        |                  |                                | C                              | 12.5  | 6.1 (48.8)   |
| 2/29   | I      | 57               | 180 IU                         | C                              | 6.8   | 2.7 (39.7)   |
|        |        |                  |                                | 1                              | 7.4   | 2.7 (36.5)   |
|        |        |                  |                                | 6                              | 3.0   | 0.7 (23.3)   |
|        |        |                  |                                | C                              | 1.9   | < 50 $\mu$ g |
|        |        |                  |                                | C                              | 5.2   | 2.1 (40.1)   |
| 7/20   | II     | 68               | 120 IU                         | 1                              | 8.7   | 4.1 (47.1)   |
|        |        |                  |                                | 6                              | 12.5  | 5.6 (44.8)   |
|        |        |                  |                                | C                              | 11.4  | 2.0 (17.5)   |
|        |        |                  |                                | C                              | 5.1   | 1.6 (31.4)   |
|        |        |                  |                                | 1                              | 20.8  | 5.6 (26.9)   |
| 11/25  | I      | 69               | 140 IU                         | 2                              | 18.2  | 5.2 (28.6)   |
|        |        |                  |                                | 6                              | 16.3  |              |
|        |        |                  |                                | C                              | 6.6   | 2.4 (36.4)   |
| 12/24  | I      | 90               | 100 IU                         | 1                              | 9.2   | 10.2 (110.9) |
|        |        |                  |                                | 2                              | 14.8  | 15.0 (101.4) |
|        |        |                  |                                | 6                              | 22.6  | 22.0 (97.3)  |
|        |        |                  |                                | C                              | 9.7   | 10.0 (103.1) |
|        |        |                  |                                | C                              | 6.4   | 2.5 (39.1)   |
| 21/26  | VI     | 65               | 100 IU                         | 1                              | 3.8   | 1.3 (34.2)   |
|        |        |                  |                                | 2                              | 2.0   |              |
|        |        |                  |                                | 6                              | 8.0   | 4.0 (50.0)   |
|        |        |                  |                                | C                              | 10.2  | 5.6 (54.9)   |
|        |        |                  |                                | C                              | 8.3   | 1.8 (21.7)   |
| 29/29  | II     | 71               | 100 IU                         | 1                              | 12.3  | 2.3 (18.7)   |
|        |        |                  |                                | 2                              | 12.1  | 3.6 (29.8)   |
|        |        |                  |                                | C                              | 7.5   | 0.3 (4.0)    |
|        |        |                  |                                | C                              | 10.2  | 2.7 (26.5)   |
|        |        |                  |                                | 1                              | 12.7  | 4.5 (35.4)   |
| 31/38  | IV     | 43               | 100 IU                         | C                              | 3.9   | 0.4 (10.3)   |
|        |        |                  |                                | C                              | 2.8   | 0.8 (28.6)   |
|        |        |                  |                                | 1                              | 7.9   | 4.1 (51.9)   |
| 37/32  | IV     | 60               | 120 IU                         | 2                              | 4.2   | 0.3 (7.1)    |
|        |        |                  |                                | C                              | 5.8   | 0.7 (12.1)   |

TABLE 5 (Continued).

| No/Age | Parity | Day of Pregnancy | Total Amount of Corticotrophin | Day of Administr. and Controls | 17 KS | D (%)      |
|--------|--------|------------------|--------------------------------|--------------------------------|-------|------------|
| 38/32  | III    | 68               | 100 IU                         | C                              | 7.6   | 3.7 (48.7) |
|        |        |                  |                                | 1                              | 6.3   | 2.7 (42.9) |
|        |        |                  |                                | 2                              | 5.2   | 1.3 (25.0) |
|        |        |                  |                                | C                              | 4.9   | 0.9 (18.4) |
| 41/34  | III    | 97               | 100 IU                         | C                              | 8.6   | 2.4 (27.9) |
|        |        |                  |                                | 1                              | 17.4  | 4.7 (27.0) |
|        |        |                  |                                | 2                              | 19.8  | 3.1 (15.7) |
|        |        |                  |                                | C                              | 5.8   | 0.1 (1.7)  |
| 42/28  | III    | 60               | 120 IU                         | C                              | 12.8  | 3.1 (24.2) |
|        |        |                  |                                | 1                              | 10.9  | 2.6 (23.9) |
|        |        |                  |                                | 6                              | 11.0  | 2.5 (22.7) |
|        |        |                  |                                | C                              | 4.1   | 0.8 (19.5) |
| 45/25  | II     | 40               | 120 IU                         | C                              | 20.4  | 5.0 (24.5) |
|        |        |                  |                                | 1                              | 17.7  | 6.4 (36.2) |
|        |        |                  |                                | 2                              | 16.0  | 5.3 (33.1) |
|        |        |                  |                                | 6                              | 14.0  | 7.6 (54.3) |
| 48/23  | I      | 75               | 100 IU                         | C                              | 14.5  | 7.6 (54.3) |
|        |        |                  |                                | C                              | 10.4  | 4.5 (43.3) |
|        |        |                  |                                | 1                              | 13.0  | 7.0 (53.8) |
|        |        |                  |                                | 2                              | 15.5  | 5.7 (36.8) |
|        |        |                  |                                | 6                              | 10.2  | 2.9 (28.4) |
|        |        |                  |                                | C                              | 3.2   | < 50 µg    |

## DISCUSSION

Total values of 17-ketosteroids do not give a full picture of the disturbance present in patients of hyperemesis gravidarum, and more detailed studies on the various components of 17-KS are necessary. The most simple procedure is to carry out the determination of dehydroepiandrosterone which is an indicator of androgen synthesis specifically after corticotrophin tolerance test. The biosynthesis of androgens takes place according to Dorfman (2) in the following way: kolesterol-pregnenolone-17-OH-pregnenolone-dehydroepiandrosterone-4androst-en-dione.

In the first trimester of pregnancy there seems to be a dysfunction of the adrenal cortex. Although the excretion of the total 17-ketosteroids is not much lower than normally is the excretion

of dehydroepiandrosterone very low. After corticotrophin stimulation increase the excretion of total 17-KS and also that of D.

The adrenocortical function of a part of patients with hyperemesis (group I) don't differ of that of nonvomiting pregnant women. Only after corticotrophin stimulation does not the excretion of D increase as much in this group as in the control group, that points out on the more severe dysfunction of the adrenal cortex in this group.

The other group of patients with hyperemesis (group II) shows quite normal adrenocortical function. The excretion of total 17-KS and D is on the same level as the corresponding excretion of non-pregnant women. Also the reaction after corticotrophin stimulation was almost normal although the rise in the excretion of D was not quite significant.

The excretion of androgens in the third group of patients with hyperemesis (group III) is quite different than that of the former groups and of normal nonpregnant and pregnant women. In this group we also find normal excretion of total 17-ketosteroids but the excretion of dehydroepiandrosterone is highly increased. It seems that the androgen synthesis (2) does not proceed beyond the D stage and D is excreted as such in urine. That can possibly be due to 3- $\beta$ -dehydrogenase deficiency. Previously the opinion prevailed that in cases with high percentage of D there is either a tumour or hyperplasia of the adrenal cortex. However, in such cases the total 17-KS excretion is high. Our series show some very high percentages of D despite normal values for total 17-KS. In these cases, however, there is *dysfunction* of the androgen production of the adrenal cortex and *not its hyperplasia*. To the dysfunction points out also that phenomenon that corticotrophin stimulation does not increase the values of 17-KS and D notably in this group of patients.

The results presented in this study cannot clarify the etiology of hyperemesis gravidarum, which probably is quite different in different cases. But the present investigation shows that in almost some cases of hyperemesis there is a definite dysfunction of the adrenal cortex that differs from the type of dysfunction present usually in early pregnancy. To shed more light on this question further studies are in progress.

## SUMMARY

Adrenocortical function in patients with hyperemesis gravidarum is studied before and after stimulation with corticotrophin by determining the excretion of total 17-ketosteroids and dehydroepiandrosterone. The 17-KS values are not lower than the corresponding values by non-vomiting women in early pregnancy and the values of nonpregnant women. The results are divided into three groups according to the relationship of the D excretion to the total 17-KS excretion. In group I as well as in the control group of non-vomiting pregnant women is the excretion of D low (1—9.9 per cent). In the control group increase the values of 17-KS and D after corticotrophin stimulation, in group I only those of 17-KS. In group II are the percentile values of D 10—19.9 per cent and on the same level as the values of nonpregnant women. In this group increase the values of 17-KS after corticotrophin stimulation. In group III the value of D in relationship to 17-KS is  $\geq 20$  per cent. It seems that there the androgen synthesis does not proceed beyond the D stage and a part of D is excreted as such. In this group there is a severe dysfunction of the adrenal cortex, the values of 17-KS and D do not increase significantly after corticotrophin stimulation.

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## MODIFICATION OF GENITAL FUNCTION AND ENDOCRINE ORGANS IN FEMALE RATS UNDER THE INFLUENCE OF RESERPINE

by

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(Received for publication July 19, 1961)

Reserpine has a variety of effects on the genital functions of animals. Dioestrus has been found to lengthen in female rats (*e.g.* 4) in the same way as during chronic morphine-sulphate therapy (11). A typical feature of the last-mentioned is the absence of corpora lutea, which is indicative of blockade of the secretion of luteinising hormone. Numerous large corpora lutea, on the other hand, have been established after reserpine therapy (1). In rabbits treated with oestradiol reserpine caused diminution of the ovaries (16) and in male rats reserpine therapy resulted in a decrease in the absolute weight of the testes (12). Reserpine has been found to delay ovulation, vaginal desquamation and menstruation in rhesus monkeys (5). No fully comparable results, however, have been recorded in clinical practice. But the galactogenous effect of reserpine is well known both in clinical (17) and in animal materials (*e.g.* 6 and 9). Lactation, however, has not been found to increase in women in the puerperium (7).

The results obtained have often been contradictory, a circumstance which it has not always been possible to account for. Tindal (1960) showed in his experiments on rabbits that different breeds of even the same animal species may respond differently to the reserpine dosage. Furthermore, therapeutic methods and doses have varied. The doses employed in animal experiments have

generally been large (as much as 1 mg/kg even) compared with the doses administered clinically. The usual clinical reserpine dose is an average of 1 mg, exceptionally perhaps 10 mg, per diem, *i.e.* c. 0.017—0.17 mg/kg and the recent tendency has been to give even smaller doses. Reserpine is recommended for clinical use also as a psychosedative in various stressful situations. It has been shown in animal experiments that psychic stimulations cause disturbances in the reproductive organs (*e.g.* 15 and 18). There seemed reason, therefore, to study the effect of reserpine on the genital functions and certain endocrine organs in test animals, using doses and a method of administration corresponding to clinical usage.

#### MATERIAL AND METHODS

The series consisted of 24 female rats of fertile age from which vaginal smears were taken both before and during the test. The smears were fixed in ether-alcohol and stained by Shorr's method (1941). A regular cycle was established in all the animals. The rats were then divided into 2 groups of 12 animals each. The rats of the experimental group were given a daily subcutaneous injection of 20  $\mu$ g of reserpine (Reserpin, Lääke Oy), *i.e.* 0.115 mg/kg, for one month. The rats of the control group were injected similarly with physiological saline. In other respects the rats lived in similar conditions. The rats of both groups were weighed and sacrificed by decapitation after 30 days. The ovaries, hypophyses and adrenal glands were prepared carefully and weighed. The ovaries of all the rats were fixed in neutral 10 per cent formalin for 24 hours, dehydrated in the usual way and embedded in paraffin. The ovaries were cut into 5  $\mu$  slices and stained according to van Gieson. The number of primary and Graafian follicles and of corpora lutea was determined for each ovary from 6 slices. The hypophyses of 4 animals in both groups were fixed, dehydrated and embedded in paraffin in the usual way. They were cut horizontally into 5  $\mu$  slices and stained with PAS-Hemalaun Orange G. The relative number of PAS-positive cells was determined as by Soiva *et al.* (1959).

»Student's» *t*-test was used in the statistical evaluation of the quantitative results. The difference between the means was considered significant when  $P$  was  $\leq 0.05$ .

## RESULTS

*Genital function* as determined from the vaginal smear changed distinctly during reserpine therapy (Fig. 1). The regular sexual cycle was disturbed and was typified by long dioestrus periods. Seven of the animals of the reserpine group had only 1—3 oestruses during the therapy of 30 days, 4 rats had 4—5 oestruses at irregular

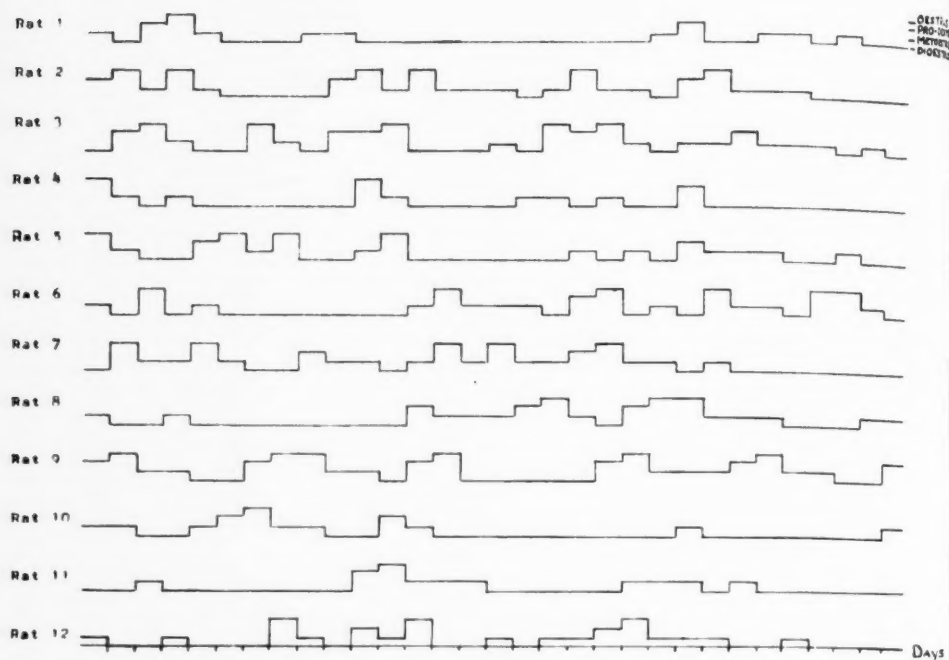


Fig. 1. — Vaginal smear samples of reserpine treated rats.

TABLE 1

EFFECT OF RESERPINE ON BODY AND ORGAN WEIGHTS

| Group             | Number of Animals | Initial Weight g | Final Weight g | Ovary mg   | p**    | Hypo-physis mg | p      | Adrenals mg | p    |
|-------------------|-------------------|------------------|----------------|------------|--------|----------------|--------|-------------|------|
| Control group..   | 12                | 173 ± 4.8*       | 198 ± 5.5      | 71.5 ± 2.9 |        | 10.8 ± 0.5     |        | 57.5 ± 2.4  |      |
| Reserpine group.. | 12                | 173 ± 6.5        | 189 ± 7.9      | 50.0 ± 3.3 | <0.001 | 8.2 ± 0.6      | <0.001 | 51.0 ± 2.6  | >0.0 |

\* Standard error

\*\* Compared with the controls

intervals and there was only 1 test animal (No. 9) in which the cycle remained practically regular.

The *weights of the rats* increased during the experimental period, in the control group slightly more than in the group treated with reserpine (Table 1).

Of the *endocrine organs* (Table 1), the weights of the ovaries and hypophyses were highly significantly lower in the reserpine than in the control group ( $P < 0.001$  in both cases). No significant difference was established in the weights of the adrenal glands.

The decrease in ovary size of the reserpine-treated rats seemed to be associated with their reduced functional activity for the quantity of corpora lutea ( $P < 0.01$ ) and primary follicles ( $P < 0.05$ ) was significantly smaller than in the controls (Table 2). The number of Graafian follicles also seemed to be smaller although there was no statistical difference.

There was no statistical difference between the groups in the number of PAS-positive cells in the hypophyses (Table 3) but they seemed to be slightly more numerous in the reserpine group. The size of PAS-positive cells seemed to be a little greater on an average

TABLE 2

MEAN NUMBER OF PRIMARY AND GRAAFIAN FOLLICLES AND CORPORA LUTEA OF OVARY PREPARATIONS IN RESERPINE-TREATED AND CONTROL RATS

| Group              | Primary Follicles | P        | Graafian Follicles | P        | Corpora Lutea | P        |
|--------------------|-------------------|----------|--------------------|----------|---------------|----------|
| Control group ..   | $5.2 \pm 0.7$     |          | $8.5 \pm 1.2$      |          | $9.5 \pm 0.9$ |          |
| Reserpine group .. | $3.7 \pm 0.4$     | $< 0.05$ | $6.5 \pm 0.7$      | $> 0.05$ | $5.5 \pm 0.9$ | $< 0.01$ |

TABLE 3

RELATIVE NUMBER OF PAS-POSITIVE CELLS IN THE HYPOPHYSES OF RESERPINE TREATED AND CONTROL RATS

| Group              | Number of Animals | Number of PAS-positive Cells, % | P        |
|--------------------|-------------------|---------------------------------|----------|
| Control group .... | 4                 | $5.4 \pm 0.8$                   |          |
| Reserpine group .. | 4                 | $6.5 \pm 1.1$                   | $> 0.05$ |

in the animals given reserpine than in the controls. Because of the great variation, no distinct difference could be demonstrated in the granulation of the cells.

#### DISCUSSION

Reserpine therapy administered to female rats of fertile age for a month in doses corresponding approximately to quantities used clinically caused a manifest disturbance of the sexual cycle. The formation of long dioestrus periods or even an acyclical phase has been assumed to be due to »pseudopregnancy»: reserpine would seem to block the function of certain hypothalamus nucleoli and induce the increased secretion of luteotrophic hormone (1). In the present work the disturbed cycle seemed, however, to be associated with atrophy of the ovaries and a decrease in the number of corpora lutea and primary follicles, *i.e.* changes which seem to suggest a decrease of ovarian function. The hypophysis weights also decreased and no differences were established in the number of PAS-positive cells. The results thus seem to suggest an at least partial weakening of the hypophysial function of gonadotrophin secretion. It must be remembered, however, that a single reserpine injection may have a different effect on the release of gonadotrophins than chronic dosage.

Conversely, both a single dose of reserpine and repeated injections cause ACTH-hypersecretion in rats (8) and function in that respect as systemic stress. Systemic stress generally causes reduced genital function and atrophy of the reproductive organs (13). The effects of psychic stress may vary. Stimulation of genital function can be established in the acute phase, but after continuous stress genital function seems to be extinguished (15). On the other hand, mere chronic audiogenic stimulation seems to cause stimulation of the gonatrophic secretion of the hypophysis (18).

The question of whether the reserpine-induced disturbance of genital function is based solely on the »systemic stress» effect of reserpine, on its effect on the catechol metabolism of the brain (3), or whether it is caused by the blockade of some centres of the hypothalamus and consequent increased secretion of luteotrophic hormone alone (1 and 2) or inhibition of luteinising hormone (10) obviously requires additional study.

## SUMMARY

Twelve rats of fertile age whose vaginal smears indicated a regular sexual cycle were given 20  $\mu$ g (= 0.115 mg/kg) of reserpine subcutaneously per day for 30 days. The regular cycle was distinctly disturbed in 11 animals, the typical feature being long dioestrus periods. The mean weights of the hypophyses and ovaries were lower in the rats given reserpine than in the controls (12 animals) and the ovaries contained fewer corpora lutea and primary follicles. The long-term administration of reserpine doses comparable to clinical doses caused dissociative changes in the reproductive organs and functions of rats.

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## EFFECT OF PROTHIPENDYLE ON LIVER PARENCHYME IN THE WHITE RAT

by

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Prothipendyle, N-(3-Dimethylaminopropyl)-thiophenylpyridylamine-HCl hydrate, has shown itself useful in the medical treatment of psychic diseases. In this respect it has much in common with phenothiazines (3, 8, 10). Its toxicity has been shown to be of less degree as compared with chlorpromazine (9, 11, 14).

In two earlier papers we have investigated the toxicity of chlorpromazine as shown by its effect on liver parenchyme in the white rat (4, 7). Similar experiments were also done with acepromazine, another phenothiazine derivative with psychiatric value (5, 6). The aim of the present study was to investigate the toxicity of prothipendyle in this same respect. The results are also compared with the above-mentioned results with phenothiazine derivatives. The methods used were similar.

### MATERIAL AND METHODS

The effect of prothipendyle on the liver parenchyme was studied by two groups of 10 white male rats. A third group of 10 rats served as controls. The animals in each group were of the same age and also in other respects the material was chosen as homogenous as possible. The drug was given subcutaneously once a day. Progressively increasing doses were used. A comparison of the literature concerning the pharmacological effectivity of prothipendyle on the one hand and chlorpromazine and acepromazine



on the other hand, showed that the firstmentioned drug is to be used in somewhat higher doses in order to obtain the same level of effectivity. Therefore, prothipendyle was given to the group I in a dosage 50% higher than had been the case in the earlier investigations with phenothiazine derivatives. On the first day 7.5 mg/kg body weight prothipendyle ('Dominal' Homburg, 'Dominex' Astra) was given and the dose was increased by this same amount daily. The medication was continued during 25 days, after this the animals were killed. In the group II rapidly increasing doses of prothipendyle were used, also in a dosage 50% higher than had been the case in the earlier investigations with phenothiazine derivatives. On the first day the dose was 30 mg/kg body weight and the dose was increased by this same amount daily. The medication was continued during 12 days, after this the animals were killed.

Each rat was weighed before the medication was started and after death, also the liver was weighed then. A specimen of the liver for microscopic study was taken immediately after death. Morphologic study took place after fixing in formol and staining with the van Gieson method. For glycogen sections were stained with the Best's carmine method. The possible occurrence of fat was studied by cutting 10  $\mu$  frozen sections and by staining them with the Scharlach-R method.

#### RESULTS

In the control group the body weight had increased by 3.1% during the treatment. In group I, receiving slowly increasing doses of prothipendyle the body weight had decreased by 16.7%. In group II, receiving rapidly increasing doses the decrease was 15.9%. In the control group the weight of the liver was 3.9% of the total body weight. At the moment of killing the weight of the liver was in the group I as well as in group II 5.5% of the body weight and 4.6% of the body weight obtained before treatment. In both experimental groups the weight of the liver, thus, had increased to an equal extent.

By studying the liver microscopically it was seen that in the group I, receiving slowly increasing doses of prothipendyle, 8 cases showed a fatty degeneration of the liver in the sections stained with Scharlach-R. On a relatively narrow zone around the central

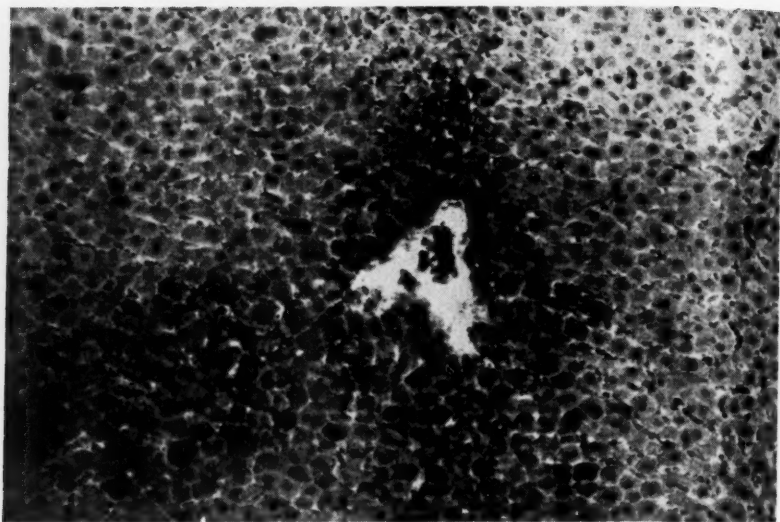


Fig. 1. — Photomicrograph of the rat liver showing fatty degeneration on a relatively small area around the central vein, caused by slowly increasing doses of prothipendyle. Scharlach-R and Weigert's haematoxyline,  $\times 280$ .

veins the liver cells were filled by small fat droplets (fig. 1). In two cases small fat droplets were seen only in some few cells here and there in the tissue. In the sections stained by the van Gieson method eight cases showed a vacuolation of the cytoplasm in the cells of the immediate surrounding of the central veins, corresponding the distribution of fat droplets in the tissue (fig. 2). In other respects the histologic picture of the liver was normal in these cases. In the ninth case the liver cells showed a vacuolar degeneration of the cytoplasm and in the tenth case the histologic picture of the liver proved to be almost normal. Sections stained by the Best's carmine method showed no essential changes in the glycogen content of the tissue.

In group II, receiving rapidly increasing doses of prothipendyle, fatty degeneration of the liver was seen in 9 cases. This, however, extended to a larger area in the liver lobule than in the group I (fig. 3). The sections stained by the van Gieson method, respectively, showed small vacuoles in the cytoplasm (fig. 4). In other respects the histologic picture of the liver proved to be normal. In the tenth case the sections stained for fat showed small fat droplets only in some cells here and there in the tissue and by the van Gieson method

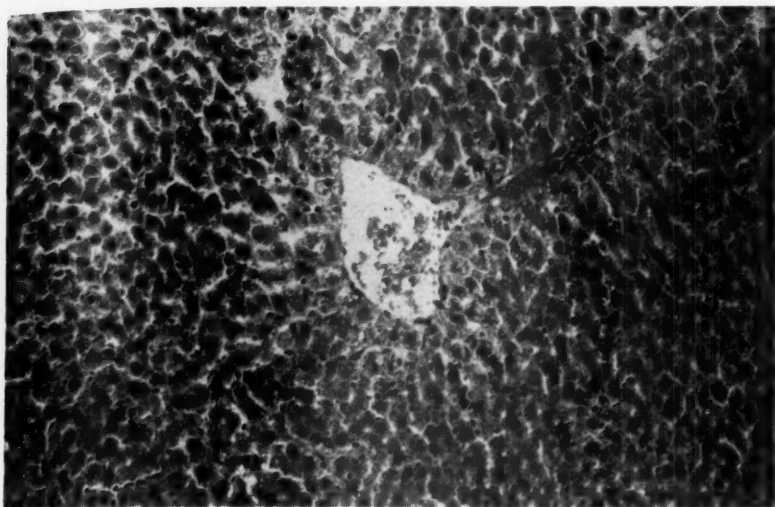


Fig. 2. — Photomicrograph showing liver parenchyma of a rat treated with slowly increasing doses of prothipendyle. A vacuolation of the liver cells in the immediate surrounding of the central vein is seen, resembling the distribution of fat droplets in the preceding photomicrograph, Van Gieson stain,  $\times 280$ .

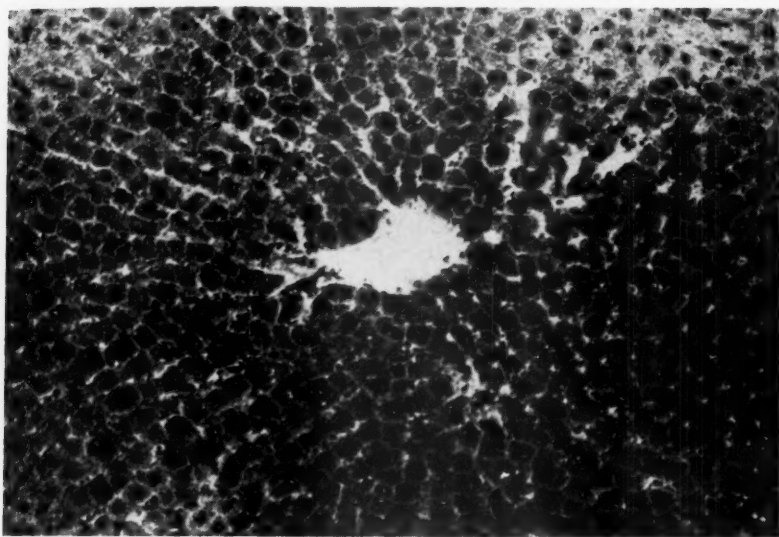


Fig. 3. — Photomicrograph of the rat liver showing fatty degeneration on a relatively wide area in the liver lobule, caused by rapidly increasing doses of prothipendyle. Scharlach — R and Weigert's haematoxyline,  $\times 280$ .

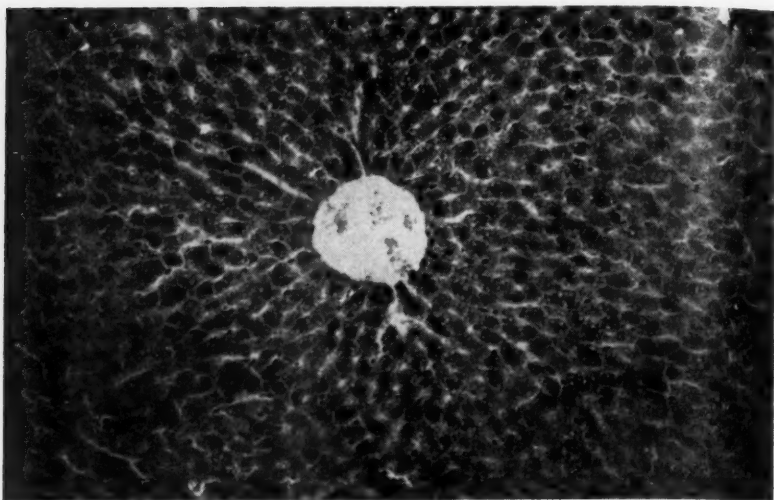


Fig. 4. — Photomicrograph showing liver parenchyme of a rat treated with rapidly increasing doses of prothipendyle. A vacuolation of the cells is seen on a relatively wide area in the liver lobule, resembling the distribution of fat droplets in the preceding photomicrograph. Van Gieson stain,  $\times 280$ .

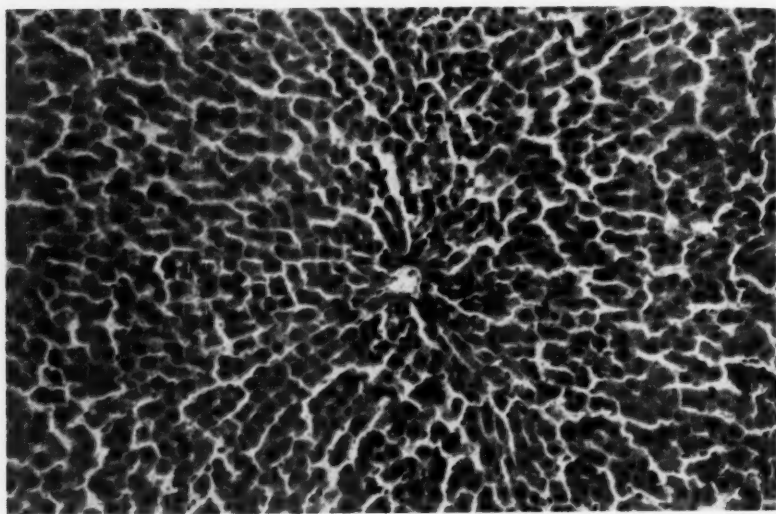


Fig. 5. — Photomicrograph showing liver parenchyme in the control group. Van Gieson stain,  $\times 280$ .

the structure of the liver was seen to be almost normal. The sections stained by the Best's carmine method showed no essential change in the glycogen content of the tissue.

#### DISCUSSION

The results exposed above show that a prolonged prothipendyle medication with large doses causes in most cases only one but rather typical parenchyme lesion of the liver in the white rat, namely fatty degeneration which appears as small fat droplets filling the cells in the centre of the liver lobules. In the earlier experiments with chlorpromazine a severe fat infiltration was seen with large fat droplets filling the liver cells at the periphery of the lobules. Extensive necroses, in addition, were seen in some cases (5, 7). Prothipendyle, on the contrary, caused fatty changes of much less degree and no necroses. In the liver lesions caused by acepromazine the main feature was a vacuolation and granulation of the cytoplasm of the liver cells, slight signs of fatty degeneration were seen in addition and small necroses in some cases (5, 6). The severity of the lesions caused by acepromazine is difficult to compare with that caused by prothipendyle because the histologic picture of the changes was different, but in both experiments the lesions were of a much milder degree than those caused by chlorpromazine.

This result shows that the toxic effect of prothipendyle on liver parenchyme in the white rat is of mild degree, especially as compared with that of chlorpromazine. This is in accordance with clinical observations showing no signs of impairment of the liver function during prothipendyle treatment (1, 2, 12, 13).

#### SUMMARY

The effect of prothipendyle on liver parenchyme in the white rat was studied in the light of two experimental series. A prolonged medication with large doses of prothipendyle caused a slight but noticeable damage of the liver parenchyme. Its microscopical signs were accumulation of small fat droplets in the cytoplasm of the cells in the centre of the liver lobules. These fatty changes were somewhat more pronounced when higher doses were used during a shorter time than vice versa when the total amount of the drug

was approximately the same. When compared with the results of earlier investigations with phenothiazine derivatives chlorpromazine and acepromazine, it was concluded that the signs of liver parenchyme damage caused by prothipendyle were of milder degree, especially as compared with the toxic effects of chlorpromazine.

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EFFECT OF NORADRENALINE AND METARAMINOLE ON  
RENAL CIRCULATION IN CATS WITH NORMAL BLOOD  
PRESSURE AND IN HEMORRHAGIC SHOCK

AN ANGIOGRAPHIC STUDY

by

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(Received for publication August 25, 1961)

A focal part phenomenon in shock is the deterioration of renal circulation. The filtration rate and renal blood flow are reduced in practically every case of shock, and there is a correlation between the decrease and the severity of the shock. The filtration fraction remains close to normal in some 19 per cent (4). When the blood pressure is 60 mmHg in man in a hemorrhagic shock the renal function ceases practically completely (3).

Vasopressor agents are the most important drugs in the treatment of hypotension. When the blood pressure rises the excretion of urine usually increases, indicating an improved glomerular filtration rate and renal blood flow. The GFR may rise during noradrenaline therapy by 30—120 cc per min. in individual cases and the RBF by 150—600 cc per min. (8). Even in the presence of renal damage, when function is further impaired by hypotension, vasopressor agents may improve renal function (9).

It is generally known, however, that noradrenaline reduces renal flow in consequence of the vasoconstriction it causes (1, 10). In man, renal vascular resistance increases by 224 per cent after the administration of noradrenaline and by 162 per cent after



metaraminole (9). Normal renal function is disturbed so much that renal flow diminishes by 711—575 cc per min. and glomerular filtration by 117—112 cc per min. after a noradrenaline injection (7).

Vasoconstriction can be very dangerous in some cases and noradrenaline administered to test animals in hemorrhagic shock has resulted in double the mortality of that among animals not given noradrenaline (2). Death has been attributed in such cases primarily to the metabolic changes originating in the internal organs during vasoconstriction. This increase in vascular resistance is hazardous also in that it may increase the number of tubular lesions during anuria and thus add to the number of permanent renal complications (6).

Following from this, the present authors decided to compare the effect of noradrenaline and metaraminole on renal blood flow both in normal test animals and in cases of bleeding shock. The investigation was conducted by means of renal angiography.

#### MATERIAL AND METHOD

The test animals were 9 adult cats which were anaesthetised with nembutal intraperitoneally, 42 mg/kg of body weight. A No. 5 Lehman catheter was introduced into the descending aorta via the exposed femoral artery. A polyethylene cannula was introduced into the femoral vein for the injection of active agents. To measure the pressure a Lehman catheter was introduced from the femoral artery of the contralateral side into the abdominal artery. The single dose was 2 ml/kg of body weight of 76 per cent Urografin (Leiras, Finland) contrast medium, introduced by means of Gidlund's automatic syringe at a pressure of 10 kg/sq.cm. An Odelca screen photography apparatus and Rapidix camera, 3 exposures per sec., was used for the roentgenography. ECG recordings with an Elema mignograph and pressure recordings with an Elema electromanometer were performed concurrently. Each test animal served as its own control. Aortography corresponding to normal conditions was the first measure to be performed. The test animals were then injected intravenously with 0.4 mg of noradrenaline and 0.5 mg of metaraminole (Aramine®) within 10 seconds in 1 ml of saline solution. Aortography was done as shown in Fig. 1 when the pressure had reached



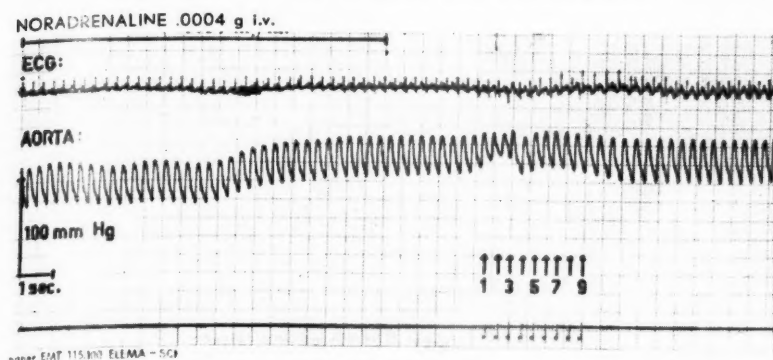


Fig. 1. — Control recording during the test. It shows the ratio between aortic pressure and the time at which the active agent is injected. Arrows 1—9 indicate the times of taking the roentgenograms during the injection of contrast medium. The injection of contrast medium causes a 4—8 mmHg rise in systolic and an ad 20 mmHg rise in diastolic pressure.

TABLE 1  
NORMAL CATS

| Agent                                    | Test 1 | Test 2 | Test 3 | Test 4 | Test 5 |
|--|--------|--------|--------|--------|--------|
| Noradrenaline<br>0.4 mg<br>intravenously | ↓<br>↓ | ↓<br>↓ | ↓<br>↓ | ↓<br>↓ | ↓<br>↓ |
| Metaraminole<br>0.5 mg<br>intravenously  | ↓<br>↓ | ↓      | ↓<br>↓ | ↓      | ↓      |

TABLE 2  
CATS IN HEMORRHAGIC SHOCK

| Agent                                    | Test 6 | Test 7 | Test 8 | Test 9 | Test 10 |
|--|--------|--------|--------|--------|---------|
| Noradrenaline<br>0.4 mg<br>intravenously | 0      | ↓      | 0      | ↓      | 0       |
| Metaraminole<br>0.5 mg<br>intravenously  | 0      | ↓<br>↓ | ↓      | ↓      | 0       |

0 = The vascular pattern of the renal cortex is nearly as good as in the control x-rays.

↓ = The vascular pattern of the renal cortex is slightly poorer than in the control x-rays.

↓ = The vascular pattern of the renal cortex is much poorer than in the control x-rays.

its maximum. Shock was caused by removing blood until the blood pressure was 50 per cent of the initial value. The shocked animals were submitted first to control angiography and then to angiographies after intravenous noradrenaline and metaraminole injections.

#### RESULTS

When the test animal's blood pressure was normal the changes established following noradrenaline injection were distinctly unfavourable compared with the control angiographies. In all cases the large arteries had narrowed, hardly any of the arteries extended to the cortical region and the contrast of the kidney shadow caused by the contrast medium was slighter (Fig. 2). In the pictures taken after the administration of metaraminole the vascular pattern of the central parts of the kidneys was more profuse than in post-noradrenaline angiographies. In 3 out of 5 cases the vascular pattern extended to the region of the renal cortex. In these cases metaraminole was injected after the noradrenaline experiment and the unfavourable effect of the angiographies does not influence the result.

One of the cats became shocked during the tests without withdrawal of blood. In this animal both noradrenaline and especially metaraminole improved distinctly the renal blood flow both in the region of the central parts of the kidneys and to some extent also in the cortical region.

Angiographies taken during hemorrhagic shock showed a manifest difference from angiographies of the same animal during normal blood pressure (Fig. 3). The large arteries were narrower in the former case and their peripheral branches in both the intestinal and renal cortical region filled more poorly and slowly than in normal conditions. The vascular structure as a whole was more vague in appearance. No distinct differences from the control angiographies were established in 3 cases after noradrenaline administered in shock. The renal blood flow was impaired to some extent in the cortical area in 2 cases. Renal hyperemia was worse after metaraminole than in the control pictures in 3 out of 5 cases and in 2 cases poorer than after the administration of noradrenaline. Hypotonia was corrected successfully by both agents.

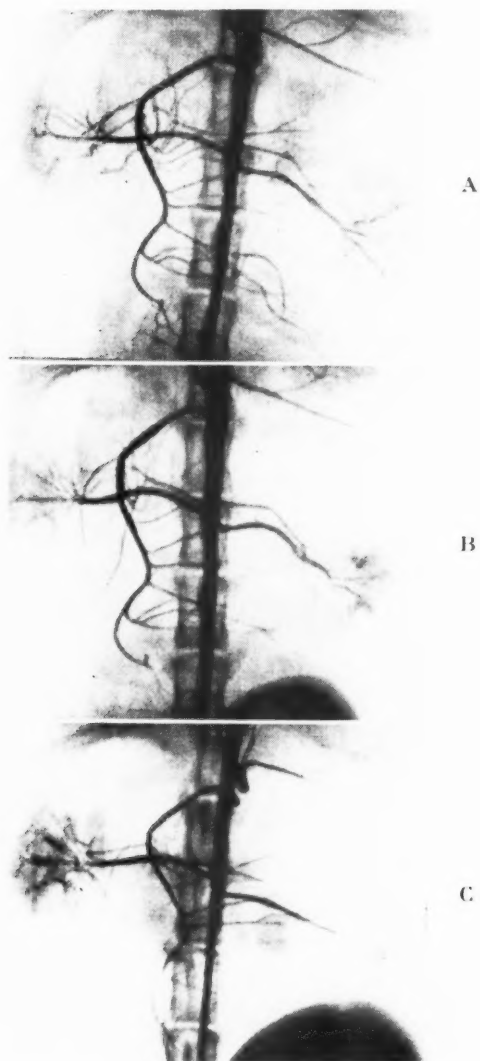


Fig. 2. — Aortography, A — in normal condition, B — during noradrenaline and C — during metaraminole effect. Impairment of the renal circulation is seen most clearly from the function of the left kidney.



Fig. 3. — Aortography A in normal condition. Individual rami of the renal artery are clearly visible as far as the cortex. In shock (B) the filling of the arteries is poorer, which appears very distinctly also in the filling of the renal arteries. Noradrenaline (C) and metaraminole (D) are not able to restore the renal circulation to normal.

#### DISCUSSION

We are aware of the difficulties of interpreting the results. Anesthesia and angiographies can as such have an untoward effect on renal circulation. Hemodynamic changes are very rapid during shock. We endeavoured to eliminate the errors by taking a great number of pictures and by changing the order of injection of the agents administered.

Noradrenaline has an unfavourable effect on the renal circulation of the test animal during normal blood pressure. This observation

is supported by earlier experimental studies of function (7, 10). The circulation is somewhat better in the kidneys during metaraminole than during noradrenaline administration, but metaraminole too impairs the renal blood flow. Livesey, Moyer and Chapman (5) showed that with greater degrees of pressor response during metaraminole administration as compared to noradrenaline there is less reduction in the renal blood flow and consequently less evidence of renal vasoconstriction. Moyer and Mills (9) established on comparing in the same patients the renal hemodynamic response to noradrenaline and metaraminole that if any difference does exist metaraminole produces a slightly greater improvement in renal function than noradrenaline.

It seems that precedence must be given in the management of hemorrhagic shock to blood transfusions and that vasopressor agents are to be combined with this therapy only in the most severe forms of shock. This would make it possible to avoid renal complications.

If shock must be treated with vasopressor agents, we prefer noradrenaline on the strength of the picture obtained by us from angiographies; it appears to be more beneficial hemodynamically. The risk of tissue necrosis, however, is considered to be greater in connection with noradrenaline than metaraminole therapy. Steroid therapy combined with this treatment may prove to be an effective supplement. If shock continues the administration of noradrenaline must be regarded as indicated whatever the risk. Some individual cases have been reported from personal clinical experience in which noradrenaline has countered the shock when metaraminole therapy has been of no avail. The improvement of renal function in consequence of controlling of the shock outweighs the danger caused by vasoconstriction. It is our contention, however, that noradrenaline should not be used too readily or for too long for the management of shock.

#### SUMMARY

Aortographic studies of the kidneys were performed on 9 cats. For five of them the aortography was done at normal blood pressure after both noradrenaline and metaraminole injection. Aortographies were performed after the injection of the same agents on 5 cats in which hemorrhagic shock was provoked.

Aortographies done before shock showed that noradrenaline caused a reduction of the renal circulation, especially in the cortical region. The adverse effect of metaraminole on the circulation was slightly smaller.

Angiographies performed during hemorrhagic shock revealed no stimulation of renal circulation after the administration of drugs. The renal vasculature was poorer after metaraminole than after noradrenaline in 2 out of 5 cases.

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## EFFECT OF SEX HORMONES ON THE ARYLSULPHATASE CONTENT OF RAT LIVER AND KIDNEY

by

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The sulphatase content of rat organs is lower before the age of sexual maturity than at sexual maturity (10). This may naturally be due solely to the phase of development. The arylsulphatase content (a-s) of human urine is at its highest at the age of 30 (1), and the enzyme content obviously does not cease to increase at the age of sexual maturity. On the other hand the activity may be affected by the changes in the quantities of sex hormones secreted with the process of evolution. Bianchi (2) noted that the administration of estrogens raises the a-s content of guinea-pig liver with NPS as the substrate and that the activity shows a direct correlation with the amount of hormone administered. The effect of androgens has not been studied. The development of the enzyme content of fetuses continues in extrauterine life in the same way as it has begun, and no major change occurs at the time of delivery (10). The quantity of hormones circulating in the fetus is nevertheless known to decrease concurrently.

The present writer studied these aspects of the effect of sex hormones by administering them *in vivo* and then analysing the enzyme content and by taking into consideration the possible effect of castration. It was possible to use crude homogenate for the rat strain examined as no metabolism of *p*-nitrophenol takes place (9, 10). The liver and the kidney are analysed earlier as their reaction to hormones with another esterase (5).

## MATERIAL AND METHODS

The series consisted of 240 Wistar rats. The a-s content of both the liver and the kidney was analysed. The material was distributed into the following 8 groups of 30 rats:

1. ordinary sexually mature male;
2. ordinary sexually mature female;
3. castrated male;
4. castrated female;
5. castrated male which received 0.1 mg of estradiolbenzoate per day for 10 days (Primogyn B oleosum Schering A.G.);
6. castrated female which also received estradiolbenzoate;
7. castrated male which received 2.5 mg of testosterone phenylpropionate (T.T.P.Organon) on the 1st and 5th day of the experiment. The analysis was made on the 10th day.
8. castrated female which also received testosterone.

All the females and males were of the same age, the former 1 year and the latter 6 months old.

The rats were killed by a blow on the neck. The tissue specimens were homogenised immediately in Potter-Elvehjem's glass homogeniser. The enzyme content was determined immediately after homogenisation.

The method employed has been described in detail elsewhere (10). P-nitrophenylsulphate was used as the substrate in a concentration of 0.001 M. This is not the optimal concentration but yields the smallest blank values. The liver was analysed at pH 7.0 and the kidney at pH 5.8, the respective optima in acetate buffer. The homogenate concentration was 1 per cent and the incubation time 3 hours. The results were calculated in  $\mu\text{g}$  of liberated nitrophenol/100 mg w/w/hr.

## RESULTS

The a-s content of the liver is given in Table 1 by age groups. The results are calculated per wet weight.

The administration of estrogens to male rats influenced the enzyme activity, the increase was statistically significant ( $P = 0.05$ ). Testosterone had a similar but weaker effect. Castration caused no change in the content. Estrogen increased the a-s content of the



TABLE 1  
ARYLSULPHATASE OF LIVER

| Group                                  | Mean $\pm$ S.E. | S.D. |
|--|-----------------|------|
| Adult male .....                       | 101 $\pm$ 4.7   | 18   |
| Castrated male .....                   | 100 $\pm$ 14    | 57   |
| Male treated with estrogen .....       | 140 $\pm$ 9.0   | 35   |
| Male treated with testosterone .....   | 128 $\pm$ 6.1   | 24   |
| Adult female .....                     | 112 $\pm$ 4.0   | 16   |
| Castrated female .....                 | 109 $\pm$ 6.9   | 27   |
| Female treated with estrogen .....     | 130 $\pm$ 8.0   | 30   |
| Female treated with testosterone ..... | 97 $\pm$ 6.6    | 24   |

TABLE 2  
ARYLSULPHATASE OF KIDNEY

| Group                                  | Mean $\pm$ S.E. | S.D. |
|--|-----------------|------|
| Adult male .....                       | 59.0 $\pm$ 1.25 | 4.8  |
| Castrated male .....                   | 45.3 $\pm$ 2.02 | 7.8  |
| Male treated with estrogen .....       | 71.8 $\pm$ 2.39 | 9.3  |
| Male treated with testosterone .....   | 56.1 $\pm$ 2.69 | 10.5 |
| Adult female .....                     | 65.3 $\pm$ 3.18 | 12.5 |
| Castrated female .....                 | 63.3 $\pm$ 2.46 | 9.6  |
| Female treated with estrogen .....     | 67.9 $\pm$ 1.54 | 6.0  |
| Female treated with testosterone ..... | 57.7 $\pm$ 2.81 | 10.5 |

liver in the females in the same way as in the males ( $P = 0.05$ ). Testosterone and castration had no effect.

The a-s content of the kidney is shown in Table 2. Castration lowered the a-s content of the male kidney statistically significantly ( $P < 0.001$ ). The administration of estradiol raised the enzyme content of the male kidney ( $P < 0.001$ ), and the female rats showed a similar tendency ( $t < 1.57$ ). Testosterone caused no significant changes in the female rats. In the male kidney on the other hand it increased the enzyme content compared with the castrated animals ( $P < 0.001$ ) but there was no difference from uncastrated male rats. The male-female difference was not calculated as the groups were not of comparable age.

## DISCUSSION

It seems probable from the results that estrogens, or at least estradiol, has an increasing effect on the a-s content of both the liver and the kidney. Reports in the literature on another esterase of the same type,  $\beta$ -glucuronidase, show the diversity that the test results may display (4, 6, 7, 12, 13). It has also been shown how much the effect of hormones is dependent on the test animal and strain used (5), *i.e.* the genetic background of the animal (8). These points must be taken into consideration in evaluating the results of studies concerned with the effect of hormones.

Castration was not found by Roy (11) to affect the difference between the sexes. He studied the enzyme of the liver. In the present investigation, too, castration did not change the enzyme content, and hence to this content there is agreement with Roy. On the other hand, a drop in activity occurred in the male kidney. Androgens thus seem to have a certain stimulating effect in males on the splitting of sulphate conjugates in the kidney. Additional proof of this is the observation that administration of testosterone to a castrated male raised the enzyme content to the level of an uncastrated male rat.

The use of crude homogenate involves the possibility that the different response of the liver and the kidney to orchietomy might be due to the fact that they contain different quantities of enzyme fractions (3). When NPS is used as the substrate it is generally fraction C that is measured. However, the pH optimum of the crude homogenate of rat liver is 7.0 and of rat kidney 5.8 in these test conditions (10). The optimum pH of pure fraction C for the rat in acetate buffer with NPS as the substrate is 7.0, and of fraction A + B 5.8. Consequently, different fractions may have been involved in these organs. The point will be clarified in a later study.

## SUMMARY

1. The arylsulphatase (a-s) activity of rat liver and kidney towards NPS under the influence of various sex hormones was studied.
2. Castration did not influence the a-s content of the liver but reduced the enzyme content of the kidney of male rat.

3. The administration of estradiol benzoate raised the a-s content both the liver and the kidney of castrated rats of both sexes.

4. The administration of testosterone did not influence the a-s activity of either the liver or the kidney of castrated females.

In male rat the administration of testosterone raised the enzyme content of the kidney in the same way as in uncastrated rats.<sup>1</sup>

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## EFFECT OF ORAL ADMINISTRATION OF NEOMYCIN AND OXYTETRACYCLIN ON URINARY EXCRETION OF THIA- MINE, RIBOFLAVIN, NICOTINIC ACID, PANTOTHENIC ACID AND BIOTIN IN MAN

by

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(Received for publication September 13, 1961)

The role of intestinal bacteria for maintenance of the vitamin economy has long been a subject of keen interest to research workers. The results obtained have been to such an extent contradictory, however, that the problem must be considered unsolved in man. Also analytical data of vitamin nutrition during intestinal sterilisation are rather scarce.

In numerous tests on animals, acceleration of growth has been achieved, as compared with the controls, by administration of various kinds of antibiotics ((14, 23, 8, 9, 17, 3, 22). Antibiotics are considered to have a so-called sparing effect on the vitamin nutrition (15, 24, 5, 4, 11, 25, 12, 1). On the other hand, with the use of insoluble sulfonamides, vitamin deficiency symptoms and stoppage of growth have been caused in test animals. The reduction in the amount of vitamins in liver and feces has been observed under the same conditions (19). Streptomycin and tetracyclin caused development of the same kind of deficiency symptoms in the test animals as in those suffering from lack of riboflavin (6).

In the present investigation, the action of long-standing administration of neomycin and oxytetracyclin on the patients' urinary excretion of thiamine, riboflavin, nicotinic acid, pantothenic acid, and biotin has been studied.

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<sup>1</sup> Financial aid has been granted by the Sigrid Jusélius Foundation.

## MATERIAL AND METHODS

The series consisted of 9 patients; three of these were women. The series was divided as follows:

*Neomycin Series.* — Two-day samples of the urine of five patients (Table 1) were collected for control before administration of neomycin (Neomycin sulphas, Lääke Oy). Neomycin was given to each patient in daily doses of 2.0 g. Two-day urine samples were then collected at intervals of one week during a period of 3—4 weeks (Table 1). On the same days, fecal samples were taken for study of *E. coli*. The patients were weighed before and after medication. The ordinary hospital diet was given during the whole test period. The urine samples were microbiologically analysed for thiamine (16), riboflavin (20), nicotinic acid and nicotinamide (21), pantothenic acid (18) and biotin (10), using *Lactobacillus*-strains as the test organism. For each analytical test a purchasable medium (Difco Laboratories Incorporated, Detroit, Michigan) was used and the methods were modified to suitable conditions in our laboratory (13).

*Oxytetracyclin Series.* — The series included two women and two men, all with pulmonary tuberculosis (Table 2). The patients were hospitalized in the sanatorium for several months before the tests, and during this period their weight had been stabilized. Thereafter for 1—6½ months, the patients were given oxytetracyclin by mouth (Terramycin, Pfizer) in daily doses of 2—4 g. Before treatment, urinary samples for control were collected only in Cases 1 and 2.

All the same analyses were performed microbiologically as mentioned before. Oxytetracyclin was not given during the two days immediately preceding collection of samples to avoid interference of a possible excretion of this antibiotic in the urine with the microbiological analysis of the vitamins.

The changes in the vitamin amount excreted was statistically assessed from the two-day values. If  $p < 0.01$  it is said that the difference is significant.

## RESULTS

*Neomycin Series.* — The results are seen in Table 1. On study of the changes in excretion values in the whole material during neomycin treatment it was noted that a statistically significant increase only in excretion of riboflavin had occurred ( $p < 0.01$ ) during a period of 3—4 weeks. The changes in excretion of the other vitamins studied were insignificant. From Table 1 it is seen that in no cases did the *Coli*-flora grow on lactose-agar after one-week neomycin treatment.

TABLE 1

EFFECT OF NEOMYCIN TREATMENT (2.0 G DAILY BY MOUTH) OVER A PERIOD OF 3-4 WEEKS ON URINARY EXCRETION OF VITAMINS B IN TEST PERSONS ( $\mu\text{g}/24$  HOURS). EACH FIGURE DENOTES THE AVERAGE CALCULATED FROM THE TWO-DAY OUTPUT

|  | Thiamine | Riboflavin | Nicotinic Acid | Pantothenic Acid | Biotin | E. coli in Feces |
|--|----------|------------|----------------|------------------|--------|------------------|
| <i>Case 1. Male, state after partial gastrectomy</i> |          |            |                |                  |        |                  |
| Before neomycin treatment .....                      | 140      | 385        | 1524           | 2936             | 9.5    | +                |
| After treatment of one week .....                    | 98       | 1223       | 1810           | 2760             | 10.5   | —                |
| " " " 2 weeks .....                                  | 109      | 674        | 1674           | 2325             | 12.6   | —                |
| " " " 3 " .....                                      | 131      | 574        | 2044           | 3430             | 8.0    | —                |
| " " " 4 " .....                                      | 119      | 487        | 1646           | 3064             | 8.0    | —                |
| <i>Case 2. Male, state after partial gastrectomy</i> |          |            |                |                  |        |                  |
| Before neomycin treatment .....                      | 185      | 798        | 531            | 3795             | 11.4   | +                |
| After treatment of one week .....                    | 163      | 1659       | 1008           | 5103             | 9.4    | —                |
| " " " 2 weeks .....                                  | 158      | —          | 1178           | 4835             | 9.2    | —                |
| " " " 3 " .....                                      | 149      | 1505       | 699            | 4865             | 11.3   | —                |
| " " " 4 " .....                                      | —        | 1593       | 895            | 5590             | 15.5   | —                |
| <i>Case 3. Male, state after partial gastrectomy</i> |          |            |                |                  |        |                  |
| Before neomycin treatment .....                      | 119      | 642        | 800            | 3097             | 8.2    | +                |
| After treatment of one week .....                    | 79       | 897        | 1031           | 2900             | —      | —                |
| " " " 2 weeks .....                                  | 109      | 330        | 961            | 3615             | 6.1    | —                |
| " " " 3 " .....                                      | 50       | 1872       | 1112           | 4693             | 10.7   | —                |
| <i>Case 4. Male, healthy person</i>                  |          |            |                |                  |        |                  |
| Before neomycin treatment .....                      | 689      | 1225       | 1254           | 5990             | 9.8    | +                |
| After treatment of one week .....                    | 258      | 1059       | 1216           | 6843             | 14.7   | —                |
| " " " 2 weeks .....                                  | 257      | 1258       | 1397           | 6843             | 17.9   | —                |
| " " " 3 " .....                                      | —        | 1322       | 1619           | 6522             | 17.3   | —                |
| " " " 4 " .....                                      | 227      | 3696       | 1309           | 6179             | 29.4   | —                |
| <i>Case 5. Female, achlorhydria</i>                  |          |            |                |                  |        |                  |
| Before neomycin treatment .....                      | 81       | 190        | 778            | 1947             | 4.2    | +                |
| After treatment of one week .....                    | 54       | 437        | 766            | 958              | 2.9    | —                |
| " " " 2 weeks .....                                  | 86       | 313        | 697            | 1495             | 2.8    | —                |
| " " " 3 " .....                                      | 64       | 254        | 921            | 1816             | 2.9    | —                |

TABLE 2

EFFECT OF OXYTETRACYCLIN (TERRAMYCIN, PFIZER) TREATMENT GIVEN BY MOUTH ON URINARY EXCRETION OF VITAMINS B IN PATIENTS WITH PULMONAL TUBERCULOSIS. EACH FIGURE DENOTES THE AVERAGE CALCULATED FROM THE TWO-DAY OUTPUT

|   | Thiamine | Riboflavin | Nicotinic Acid | Pantothenic Acid | Biotin |
|---|----------|------------|----------------|------------------|--------|
| <i>Case 1. Male</i>                         |          |            |                |                  |        |
| Before oxytetracyclin treatment .....       | 190      | 643        | —              | 4350             | 7.3    |
| After treatment of one month (4.0 g/die) .. | 290      | 736        | 1376           | 5257             | 19.6   |
| <i>Case 2. Female</i>                       |          |            |                |                  |        |
| Before oxytetracyclin treatment .....       | 201      | 1800       | 1018           | 5867             | 20.1   |
| After treatment of three months (2.0 g/die) | 282      | 2144       | 2548           | 6002             | 11.9   |
| <i>Case 3. Male</i>                         |          |            |                |                  |        |
| After treatment of two months (2.0 g/die)   | 207      | 2003       | 739            | 5828             | 8.7    |
| <i>Case 4. Female</i>                       |          |            |                |                  |        |
| After treatment of 6 ½ months (4.0 g/die)   | 227      | 1060       | 2169           | 6338             | 5.0    |

*Oxytetracyclin Series.* -- Table 2 shows the effect of oxytetracyclin on the urinary excretion of vitamins B. Cases 1 and 2 reveal that oral administration of oxytetracyclin did not change the vitamin excretion to any considerable extent. Only in Case 2 the increase in excretion of nicotinic acid during three months was statistically significant ( $p < 0.01$ ). Study of the biotin excretion revealed diverging results in that the excretion in one patient (Case 1) increased significantly and decreased significantly in the other (Case 2).

In Cases 3 and 4, there was no possibility to take urine samples before starting oxytetracyclin treatment. On comparison of the analytical results with the average normal values obtained in a Finnish material (13) it is seen that excretion of all the vitamins studied, except biotin, was well within the normal borders. The biotin excretion values were considerably below the average normal level. In Cases 3 and 4, corresponding determinations were made three months later and a slight decrease was recorded in the urinary excretion of all the vitamins studied, except in biotin excretion in which an increase occurred.

## DISCUSSION

Formerly sulfonamides were considered to cause vitamin deficiency most closely because they destroy the intestinal bacterial flora. During administration of antibiotics, symptoms in the mucous membranes are also sometimes observed; these improve rapidly, however, on cessation of intake of the drug. Symptoms of this kind appear sometimes almost immediately after starting the antibiotic treatment. There seems to be several vitamin B depots in the organism, the exhaustion of which may be studied in connection with various deficiency diets. It has been observed that the vitamin content of the excreta or of body fluids is not in general exhausted suddenly, but slowly, during the course of some weeks or months. It thus seems surprising that treatment with antibiotics would cause the arising of vitamin deficiency symptoms in a few days, especially when the exogenous supply of vitamins is satisfactory. Also in blood analyses of patients with the mucous membrane symptoms have been obtained vitamin values comparable with completely normal ones at our hospital.

From our results one may conclude that:

Also the long-standing administration of neomycin and oxytetracyclin has no marked effect on the urinary excretion of vitamins B as a whole. Only in case of riboflavin excretion there is a clear increase in the output.

This again, can be in connection with the s. c. vitamin sparing effect of antibiotics and with the observation (2, 9), that some antibiotics have the capacity to accelerate the growth and increase the body weight.

## SUMMARY

The effect of oral administration of neomycin and oxytetracyclin on the urinary excretion of thiamine, riboflavin, nicotinic acid, pantothenic acid and biotin was tested on 6 men and 3 women.

1. Neomycin was considered to have an increasing effect during 3—4 weeks on the excretion of riboflavin.

2. Oxytetracyclin treatment caused no several decrease in the urinary excretion of vitamins B, in spite of the treatment covering more than six months in one instance. Contradictory, there occurred normal excretion values or a slight tendency to increased output.



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## EFFECT OF RESERPINE ON THE PREGNANCY AND FETUS OF RAT

by

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It has been shown experimentally that reserpine influences the organism in many ways. As regards the gonadal function, reserpine has induced disturbances in the estrus cycle of rat and reduced conception (5). Barraclough and Sawyer (2) succeeded in blocking the normal estrus cycle of the rat by means of reserpine. Reserpine affected both the ovulation and menstruation of Rhesus monkeys, too (4). Reserpine retarded the development of the genitals and caused atrophy of the testes, the seminal vesicles and the prostate in growing mature male rats (7).

The effect of reserpine on the lactiferous gland and the secretion of milk has been demonstrated in animal experiments, *e.g.* in rabbit (9) and rat (3, 8, 6). There have been fewer studies of the effect of reserpine on the fetus. A decrease in the number of viable young after reserpine treatment was observed in the investigation by Gaunt *et al.* (5), but lactation and the birth weights of the young were not affected. Adams and Hirschinson (1) injected Serpasil into the yolk of hen eggs during the incubation and noted retarded growth and development of the embryos.

Reserpine is used extensively for elevated blood pressure during pregnancy and for toxemias of pregnancy. The purpose of the present work was to study the effect of reserpine on the course of

<sup>1</sup> Aided by a grant from the Sigrid Jusélius Foundation.

the pregnancy in rats and especially on the development of the young when reserpine treatment is instituted at the beginning of pregnancy.

#### MATERIAL AND METHODS

The test animals were 33 Long-Evans strain female rats aged 4 months and weighing 146—250 g. Five female rats were housed together with one male. When vaginal samples indicated the termination of the estrus cycle, reserpine injections were started and the females were isolated. Eight rats were given 5  $\mu$ g of reserpine per 100 g of body weight subcutaneously once a day (group A), 8 received 15  $\mu$ g (group B) and 10 received 30  $\mu$ g (group C). Seven control rats were given a corresponding quantity of saline solution (group D).

After the birth of the young they were weighed and sacrificed. Gonads, adrenals, liver and heart were taken from one male and one female in each litter and fixed in 10 per cent formalin. The ovaries were stained by van Gieson's method and the testes, livers and hearts with hematoxylin-eosin.

#### RESULTS

During the treatment, 5 of the female died in the highest dosage group (group C). No noteworthy differences were established between the different groups in the duration of pregnancy. The average period during which the animals of the various groups received injections was as follows: Group A 18 days, group B 15 days, group C 15 days and group D 14 days.

The number of viable young was greatest, 69 in all, in the control group and smallest, 36, in the highest reserpine dosage group. The number of the young per litter in both groups was equal different 9.5 and 7.2 (Table 1). Groups A and B had average litters of 7.3 and 8.3 young.

The number of stillborn or young that died immediately after birth was considerable in group C, *i.e.* 14, compared with the other groups (Table 1). Groups A and D both included one dead and group B, in which the mothers received the second largest dosage of reserpine, had 5 dead.

TABLE 1

| Group                  | Mothers,<br>Number | Viable<br>Young,<br>Number | Mean Number of<br>Viable Young<br>per Litter | Dead    |       |
|------------------------|--------------------|----------------------------|--|---------|-------|
|                        |                    |                            |  | Mothers | Young |
| A ( 5 $\mu$ g) . . . . | 8                  | 58                         | 7.3  | —       | 1     |
| B (15 $\mu$ g) . . . . | 8                  | 66                         | 8.3  | —       | 5     |
| C (30 $\mu$ g) . . . . | 5                  | 36                         | 7.2  | 5       | 14    |
| D (control) ..         | 7                  | 69                         | 9.5  | —       | 1     |

TABLE 2

| Group | Mean Weight of Young<br>P |        | Liver weight<br>Body Weight | Heart Weight<br>Body Weight |
|-------|---------------------------|--------|-----------------------------|-----------------------------|
| A     | 6.20 $\pm$ 0.09           |        | 0.57                        | 0.06                        |
| B     | 5.88 $\pm$ 0.05           | < 0.01 | 0.54                        | 0.06                        |
| C     | 5.09 $\pm$ 0.17           | 0.001  | 0.54                        | 0.06                        |
| D     | 6.14 $\pm$ 0.08           |        | 0.55                        | 0.06                        |

The average weight of the viable young was highest in the control group and group A (Table 2), significantly smaller in groups B and C. The ratio between heart weight and body weight, on the one hand, and between liver weight and body weight on the other, was nearly the same in the different groups.

Microscopic examination revealed no differences between the organs in question. Neither developmental disturbances nor malformations were demonstrated.

#### DISCUSSION

The dosage given to group C during the experiment was obviously toxic and killed 5 mothers. Not a single mother died in groups A and B.

As regards the number of young, the result concurs with that obtained by Gaunt *et al.* (5), *i. e.* the experimental groups had

fewer liveborn young than the control group. However, no regularity was observed in this respect, and the litters of the 2 higher dosage groups were not distinctly smaller than those of the group given the smallest dosage. — There were considerable differences between the groups in the number of dead young (Table 1). Group A, in which the reserpine dosage was the smallest and relatively closest to the therapeutic dosage for man, had the same number of dead young as the control group. In group B the number of deaths was greater, 5 in all, and it was greatest, 14, in group C. It seems that in sufficiently large quantities reserpine reduces the viability of the young.

It will be seen from Table 2 that the mean birth weights of the young in groups B and C different significantly from the control birth weight. The investigation by Gaunt and his co-workers (5) showed no difference in the birth weights of the young, but the amount of reserpine administered was greater in the present work, Adams and Hirschinson (1), on the other hand, observed a reduction in the weights of chickens and retardation of development following Serpasil treatment. Development was slowed down especially in the liver, heart and forelimb. It was not possible to make the same observation in the organs examined in the present investigation. This can be seen in Table 2: the heart weight: body weight and liver weight: body weight ratios were nearly the same in the different groups. Reserpine obviously had a different effect on the developing organism in the study by Adams and Hirschinson (1). This is understandable as they injected Serpasil direct into the yolks.

The present work established that if reserpine is injected into female rats during pregnancy the birth weight and viability of the young are reduced. It is not possible, however, to state whether this is caused by the direct effect of reserpine on the fetus or whether it is possibly the consequence of a general deterioration in the condition of the mothers.

#### SUMMARY

The effect was studied on the pregnancy and fetus of rats of a daily subcutaneous injection of reserpine into the mother after the beginning of pregnancy. The reserpine doses were 5, 15 and 30  $\mu$ g per 100 g of body weight. The results were:

1. The number of viable young per litter was smaller in the groups given reserpine than in the control group.

2. The numbers of dead young were greater in the 2 groups given larger doses of reserpine than in the low-dosage and control groups.

3. The birth weights of the young in the 2 groups given larger reserpine doses were significantly smaller than the control birth weights.

4. Neither retardation of development nor developmental disturbances were demonstrated histologically in the gonads, adrenals, liver and heart.

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## INFLUENCE OF IPRONIAZID AND 5-HYDROXYTRYPTOPHAN ON THE 5-HYDROXYTRYPT- AMINE CONTENT IN THE GASTRO-INTESTINAL TRACT AND THYROID GLAND OF RATS

by

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(Received for publication October 5, 1961)

It is known that the monoamine oxidase inhibitor iproniazid increases the amount of 5-hydroxytryptamine (5HT) in some tissues, like brain (7, 8, 10, 13) and blood platelets (11). In several animal species, the largest body depot of 5HT is the intestinal mucosa. Iproniazid has also been found to increase the content of 5HT in the small intestine of guinea pigs but not in those of rats and rabbits (9, 10). Because the possible changes taking place only in parts of the gastro-intestinal tract might remain undiscovered when the whole organs are extracted, we analysed various parts of the small intestine and stomach after iproniazid treatment. The effect of iproniazid on the 5HT in thyroid gland, phylogenetically related to alimentary tract and known to contain high concentrations of this amine in rats (6), was also measured in some animals. Administration of the precursor of 5HT, 5-hydroxytryptophan (5HTP), also increases the 5HT content in some tissues, especially in the brain (1, 2, 7, 14). After iproniazid administered alone had failed to increase 5HT in the experiments to be reported, 5HTP was employed with and without iproniazid pretreatment.

## METHODS

Male Sprague-Dawley rats of the average weight of 150 g were used. They were kept on standard diet without fast during the experiments, which were made at room temperature of about 22°C. Iproniazid phosphate (F. Hoffmann — La Roche & Co., Basel) was injected subcutaneously at a dose of 100 mg/kg of the base. 1% solution of dl-5HTP (F. Hoffmann — La Roche & Co., Basel) was made in 0.01 N-HCl and 100 mg/kg dose was injected intraperitoneally. Further details of the treatment are given in the text.

The animals were killed by decapitation and the following tissues were dissected: Fundus (cardiac part) of the stomach (approx. weight 400 mg), pyloric (glandular) part of the stomach (about 600 mg), duodenum or upper part of the small intestine (about 900 mg, beginning 0.5 cm from the pyloric sphincter), ileum or lower part of the small intestine (about 600 mg, beginning 0.5 cm cranially from the ileo-coecal junction) and thyroid glands.

The thyroid glands were extracted with 90% acetone (6). The extraction of the gastro-intestinal tissues, while studying the effect of iproniazid alone, was done with acetone and heptane, as previously described (8). After these extractions 5HT was assayed biologically by using the rat stomach method (16). When 5HTP was used the gastro-intestinal tissues were analysed with spectrophotofluorometric method (15). To remove 5HTP the butanol extracts were washed three times with borate buffer. The 5HT doses refer to the base.

## RESULTS

*Iproniazid.* — The results obtained by injecting iproniazid alone are given in table 1. The thyroid glands of iproniazid-treated animals contained a little but not significantly more 5HT than the glands of the controls. Generally the amounts of 5HT found in the gastro-intestinal tract of iproniazid rats were lower than in the corresponding controls. The differences, however, were of no significance. The fundus of the rat stomach had 1 µg/g or less of 5HT, i.e. about one tenth of the concentrations found in the other gastro-intestinal tissues analysed (biologically).

*5HTP.* — The results obtained when 5HTP was injected alone are presented in the first part of table 2. The administration of 5HTP failed to increase the 5HT concentration in the thyroid glands. In the fundus and pylorus of the stomach, and especially in the duodenum, some higher values were found. The increase was not regular and was usually less than 100%. The time and response relationship was not clear. On the whole, the results reflect great



EFFECT OF IPRONIAZID ON THE 5HT CONTENT IN VARIOUS TISSUES OF RATS. THE MEANS (NUMBER OF ANIMALS)  $\pm$  S.E. OF THE MEAN ARE GIVEN. ACCORDING TO STUDENT'S T-TEST NONE OF THE EXPERIMENT VALUES IS SIGNIFICANTLY ( $p < 0.05$ ) DIFFERENT FROM THE CORRESPONDING CONTROL VALUE

| Treatment  | 5-Hydroxytryptamine $\mu\text{g/g}$ of Fresh Tissue |                       |                      |                       |                     |
|--|---|-----------------------|----------------------|-----------------------|---------------------|
|  | Thyroid Gland                                       | Stomach               |                      | Small Intestine       |                     |
|  |   | Fundus                | Pylorus              | Duodenum              | Ileum               |
| Iproniazid 100 mg/kg<br>16hr. before killing<br>4 days before killing<br>4 $\times$ 100 mg/kg <sup>1</sup> | 8.03 (9) $\pm$ 1.22                                 | 0.678 (9) $\pm$ 0.060 | 10.00 (5) $\pm$ 1.37 | 5.51 (10) $\pm$ 0.672 | 8.07 (7) $\pm$ 1.10 |
|  | 8.54 (9) $\pm$ 1.50                                 | 0.681 (7) $\pm$ 0.110 | 8.56 (5) $\pm$ 1.58  | 4.79 (7) $\pm$ 0.181  | 7.55 (6) $\pm$ 1.40 |
|  |   | 0.522 (4) $\pm$ 0.085 |                      | 4.10 (4) $\pm$ 0.85   |                     |
|  |   | 0.500 (4) $\pm$ 0.126 |                      | 4.18 (4) $\pm$ 0.375  |                     |

<sup>1</sup> 100 mg/kg daily for 4 days, last injection 16 hr. before killing.

TABLE 2

EFFECT OF 5-HYDROXYTRYPTOPHAN (5HTP) AND IPRONIAZID (16 HOURS BEFORE 5HTP) PLUS 5HTP ON THE 5HT CONTENT IN VARIOUS TISSUES OF RATS. THE MEANS (NUMBER OF ANIMALS)  $\pm$  S.E. OF THE MEANS ARE GIVEN. P IS GIVEN ONLY WHEN THE VALUE IS SIGNIFICANTLY ( $p < 0.05$ ) DIFFERENT FROM THE CORRESPONDING CONTROL

| Treatment  | 5HTB<br>Hours before<br>Decapitation | 5-Hydroxytryptamine $\mu\text{g/g}$ of Fresh Tissue |                    |                      |                      |  |
|------------|--------------------------------------|---|--------------------|----------------------|----------------------|--|
|            |                                      | Thyroid Gland                                       | Stomach            |                      | Duodenum             |  |
|            |                                      |   | Fundus             | Pylorus              |                      |  |
| Iproniazid | 100 mg/kg 1hr                        | 6.0 (3) $\pm$ 0.9                                   | 1.0 (4) $\pm$ 0.2  | 6.1 (4) $\pm$ 1.5    | 3.3 (4) $\pm$ 0.3    |  |
|            |                                      | 6.0 (3) $\pm$ 1.0                                   | 1.3 (4) $\pm$ 0.4  | 7.6 (3) $\pm$ 0.8    | 7.2 (4) $\pm$ 1.2    |  |
|            | 2<br>4<br>7                          | 5.9 (3) $\pm$ 0.6                                   | 1.2 (4) $\pm$ 0.4  | 7.6 (4) $\pm$ 1.5    | 4.0 (4) $\pm$ 0.9    |  |
|            |                                      | 4.8 (3) $\pm$ 1.1                                   | 1.6 (4) $\pm$ 0.03 | 9.2 (3) $\pm$ 1.2    | 6.9 (4) $\pm$ 1.2    |  |
| 100mg/kg   | 100 mg/kg 1hr                        | 7.1 (4) $\pm$ 0.7                                   | 1.0 (3) $\pm$ 0.1  | 7.0 (3) $\pm$ 1.5    | 3.3 (3) $\pm$ 0.4    |  |
|            |                                      | 21.0 (2) (18.4, 23.6)                               | 0.9 (4) $\pm$ 0.2  | 5.5 (4) $\pm$ 0.5    | 4.1 (4) $\pm$ 1.2    |  |
|            | 2<br>3                               | 24.9 (5) $\pm$ 3.1                                  | 2.2 (3) $\pm$ 0.2  | 8.6 (3) $\pm$ 0.7    | 17.0 (3) $\pm$ 2.3   |  |
|            |                                      | p < 0.01  | p < 0.01           | p < 0.02             | p < 0.01             |  |
|            |                                      | 29.3 (2) (28.5, 30.0)                               | 2.2 (4) $\pm$ 0.6  | 10.7 (4) $\pm$ 2.0   | 12.7 (4) $\pm$ 4.0   |  |
|            |                                      |   |                    | p < 0.05             |                      |  |
|            |                                      |   | 1.8 (2) (1.5, 2.2) | 10.2 (2) (8.7, 11.7) | 14.5 (2) (6.5, 22.5) |  |

differences in the response of individual animals to the injection of 5HTP. It is unlikely that 5HTP itself interfered in the analysis. The addition of one mg of 5HTP to intestinal tissue homogenate before the extraction did not increase the result.

*5-HTP with Iproniazid.* — The second part of table 2 shows that in iproniazid-pretreated rats, 5HTP produced more pronounced 5HT increase in all tissues studied. This combination of treatment proved fatal within three hours for a number of rats.

The increase of 5HT was from two- to four-fold in the thyroid glands and in the fundus. Percent-wise it was less in the pylorus than in the fundus. However, in absolute terms, the increase was more in the pylorus than in the fundus. Duodenum showed a pronounced elevation of 5HT values and the mean increase was from three- to four-fold.

#### DISCUSSION

Iproniazid failed to increase the 5HT content in any of the rat's gastro-intestinal tissues or thyroid glands. This is contrary to what we were led to expect from previous experiments where duodenal pieces were analysed (8). Iproniazid is known to inhibit 5HTP-decarboxylase *in vitro* (17). In those of our experiments in which four days were allowed for the single iproniazid dose to act, the amine content showed a further decrease.

If this decrease was due to the decarboxylase inhibition, the inhibition is long-lasting and not dependent on the presence of unmetabolised iproniazid. Iproniazid disappears from most tissues within 24 hours, but the monoamine oxidase inhibition lasts for several days (4). In mice, iproniazid treatment has also been found to lower the catechol amine content in suprarenals, heart, liver and spleen (12).

5HTP, especially when given after iproniazid pretreatment, produced an elevation of 5HT in all the tissues studied. The rat intestine has been reported to be devoid of monoamine oxidase activity (3). However, the present finding agrees with our *in vitro* (not published) experiments, in which the 5HTP decarboxylase activity of rat's tissues was studied. The addition of a monoamine oxidase inhibitor, pheniprazine ( $10^{-5}$  or  $10^{-4}$ ), increased the amount of 5HT synthesised by the fundus and duodenum by about 100%.

The same happened when the thyroid glands of rats and other animals were studied.

In rabbits, the mucosa of the fundus part of the stomach contains as much or even more 5HT than the mucosa of the pyloric part of the stomach (18). It is of interest that the rat fundus was found to have much less 5HT than the pylorus. Another difference between these two parts is that reserpine releases 5HT from the mucosa of the prepyloric part of the stomach but not from the fundus in rabbits (18). These findings indicate that the storage mechanisms of the two parts for 5HT may be different. Both parts, however, were able to increase their amine content in the present experiments although the increase in  $\mu\text{g/g}$  was greater in the pylorus. This agrees with the *in vitro* finding (not published) on the 5HTP-decarboxylase. It should be mentioned that 5HTP administration can increase the 5HT content to a high level in the rat uterus (1), which normally contains very little if any of 5HT.

Factors regulating the 5HT content in tissues include, in addition to the 5HTP decarboxylase activity and 5HT inactivation, the capacity of the tissue to hold 5HT in its binding sites and the amount of 5HTP available for decarboxylation. Neither inhibition of the oxidative deamination nor excess of the precursor was able alone to increase the 5HT content in the tissues studied. It is likely that for maintaining a constant 5HT level the cellular binding mechanisms are most fundamental (5).

#### SUMMARY

Monoamine oxidase inhibitor, iproniazid, and the precursor of 5-hydroxytryptamine (5HT), 5-hydroxytryptophan (5HTP) were administered to rats, and their influence on the 5HT content of thyroid gland and various parts of the gastrointestinal tract was studied by using biological and chemical methods.

Monoamine oxidase inhibition or administration of the precursor alone had little or no effect on the 5HT content of the organs studied.

Administration of 5HTP after iproniazid pretreatment induced a rapid accumulation of 5HT. In the thyroid gland, the rise of 5HT concentration was pronounced, and values up to 4–5 times higher than in the untreated or either iproniazid- or 5HTP-treated controls

were found. From the gastrointestinal tissues, duodenum showed the highest and fundus of the stomach the lowest ability to increase its 5HT content. The fundus part of stomach also normally contained less 5HT than pyloric part or duodenum. The factors contributing to the results were discussed.

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## THE PHAGE TYPES OF SALMONELLA PARATYPHI B

### REPORT ON 357 HUMAN CASES

by

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(Received for publication March 29, 1961)

In the phage typing of *Salmonella paratyphi* B strains the Felix and Callow method has been used which has been obtained from the International Reference Laboratory for Enteric Phage Typing of the English Public Health Laboratory Service. The phages used have been: 1, 2, 3a, 3b, Jersey, Beccles, Taunton, BAOR, Dundee, 1010 and anti-O. Phage 3aI has not been used.

Out of each bacteriologically found human paratyphoid B case the phage typing of the strain has been carried out once. The typing has consisted of the strains of the cases diagnosed by the State Serum Institute and the Municipal Bacteriological Laboratory, Helsinki, Department of Medical Microbiology, University of Turku, and provincial bacteriological laboratories, Lappeenranta, Kuopio, Oulu<sup>1</sup>). Thus the laboratories cover almost the whole of Finland as regards the diagnosis of the human cases. However, from the area of Vaasa province no samples have been obtained and thus on the basis of this study a correct picture cannot be formed as regards this area. During the period 1. 12. 59 — 28. 2. 61 the phage typing of the *Salmonella paratyphi* B strain of 357 patients has been carried out. The results are shown in Table 1 giving the number of the cases and how many districts the type in question has appeared.

The most common *Salmonella paratyphi* B phage type during the study period has been Taunton. There have been 258 strains

<sup>1</sup>) We wish to thank the directors of the laboratories in question for their interest in the matter and for their trouble in sending the strains.

TABLE 1

THE PROVINCIAL DISTRIBUTION OF DIFFERENT SALMONELLA PARATYPHI B PHAGE TYPES IN 35  
CASES DURING THE PERIOD 1. 12. 59—28. 2. 61. CASES/LOCALITIES

| Province            | 3a           | 3a var. |       |     | Jersey | Taunton | Dundee | Beccles | NST | NT  |
|---------------------|--------------|---------|-------|-----|--------|---------|--------|---------|-----|-----|
|                     |              | 1       | 2     | 5   |        |         |        |         |     |     |
| Uusimaa .....       | —            | 1/1     | 3/3   | —   | —      | 16/6    | 1/1    | 1/1     | 1/1 | 1/1 |
| (Helsinki) ....     | —            | (1)     | (1)   | —   | —      | (8)     | —      | (1)     | (1) | (1) |
| Turku-Pori .....    | —            | 6/3     | 15/10 | —   | 1/1    | 20/9    | 1/1    | —       | 1/1 | 1/1 |
| (Turku) .....       | —            | —       | (5)   | —   | —      | (3)     | —      | —       | —   | —   |
| Ahvenanmaa ....     | —            | —       | —     | —   | —      | —       | —      | —       | —   | —   |
| Häme .....          | —            | 2/2     | 5/3   | —   | —      | 36/10   | 1/1    | 1/1     | —   | 1/1 |
| (Tampere) .....     | —            | (1)     | —     | —   | —      | —       | —      | (1)     | —   | —   |
| Kymi .....          | —            | —       | 11/6  | —   | —      | 5/4     | —      | 1/1     | —   | —   |
| Mikkeli .....       | 1/1          | 1/1     | 5/4   | —   | —      | 10/7    | —      | —       | 1/1 | —   |
| Kuopio .....        | —            | 2/2     | 1/1   | —   | —      | 105/7   | —      | —       | —   | 1/1 |
| Pohjois-Karjala ..  | —            | 9/1     | 10/2  | —   | —      | 9/4     | —      | 1/1     | 1/1 | —   |
| Vaasa .....         | (No samples) |         |       |     | —      | —       | —      | —       | —   | —   |
| Keski-Suomi ....    | —            | —       | —     | 1/1 | —      | 42/4    | —      | —       | 2/2 | 1/1 |
| Oulu .....          | —            | 1/1     | 2/1   | —   | —      | 7/2     | —      | 1/1     | —   | 1/1 |
| Lappi .....         | —            | 1/1     | 1/1   | —   | —      | 8/4     | —      | —       | —   | —   |
| Finland, total .... | 1/1          | 23/12   | 53/31 | 1/1 | 1/1    | 258/57  | 3/3    | 5/5     | 6/6 | 6/6 |

(72%) of this type out of the examined material and it has appeared in 57 different districts. This type has been found in all the provinces apart from Vaasa province from where no strains have been obtained for phage typing. Three more extensive epidemics have taken place the cause of which has been of phage type Taunton. The next most common phage types have been 3aI var. 2 (53 strains, 18%) and 3aI var. 1 (23 strains, 8%) which also have appeared almost all over the country. Also they have been found to have caused some smaller epidemics. In addition five other phage types have been discovered as scattered cases: Beccles (5 cases), Dundee (3 cases), and 3a, 3a var. 5, Jersey (one case each). About 3% of the strains belong to groups that cannot be specifically typed (NST) (6 strains) with the method or that cannot be typed (NT) (6 strains).

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# THE PHAGE TYPES OF SALMONELLA TYPHI MURIUM

REPORT ON 504 HUMAN CASES

by

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In the phage typing of *Salmonella typhi* murium strains the Felix and Callow «old scheme» added with type 5 has been used which method has been obtained from the International Reference Laboratory for Enteric Phage Typing of the English Public Health Laboratory Service. The typing phages used have been: 1, 1a, 1a var. 1, 2, 2a, 2b, 2c, 2d, 3, 3a, 4, and 5.

Out of each bacteriologically found human *Salmonella typhi* murium case the phage typing of the strains has been carried out once. The phage typing has consisted of strains isolated by the State Serum Institute and the Municipal Bacteriological Laboratory, Helsinki, Department of Medical Microbiology, University of Turku, and provincial bacteriological laboratories, Lappeenranta, Kuopio, Oulu<sup>1</sup>). Thus the laboratories cover almost the whole of Finland. As regards Vaasa province the study does not give a correct picture because no strains have been obtained from the laboratory in this area.

During the period 1. 12. 59 — 28. 2. 61 the *Salmonella typhi* murium strain of 504 patients has been phage typed. The results are shown in the table showing the number of cases by provinces and in how many districts the type in question has appeared.

Out of the examined strains it has been possible to phage type 489 of them and they divided into 8 different types. It has not been

<sup>1</sup>) We wish to thank the directors of the laboratories for co-operation.



THE PROVINCIAL DISTRIBUTION OF DIFFERENT *SALMONELLA* TYPHI MURIUM PHAGE TYPE IN 504 CASES DURING THE PERIOD 1. 12. 59 — 28. 2. 61. CASES/LOCALITIES

| Province             | 1     | 1a     | 1a<br>var. 1                           | 2   | 2a  | 2b    | 2c  | 3a  | NT   |
|----------------------|-------|--------|--|-----|-----|-------|-----|-----|------|
| Uusimaa .....        | 5/2   | 53/6   | 9/3                                    | 2/1 | 1/1 | 23/4  | 2/2 | —   | 11/3 |
| (Helsinki) .....     | (4)   | (42)   | (7)                                    | (2) | (1) | (18)  | (1) | —   | (9)  |
| Turku-Pori .....     | 3/2   | 267/32 | 2/1                                    | 2/2 | 1/1 | 4/2   | —   | —   | 3/2  |
| (Turku) .....        | (1)   | (134)  | —                                      | (1) | —   | (3)   | —   | —   | (1)  |
| Ahvenanmaa .....     | —     | 1/1    | —                                      | —   | —   | —     | —   | —   | —    |
| Häme .....           | 1/1   | 7/5    | 2/1                                    | —   | —   | 1/1   | 1/1 | —   | 1/1  |
| (Tampere).....       | (1)   | (2)    | (2)                                    | —   | —   | (1)   | —   | —   | (1)  |
| Kymi.....            | 3/2   | 2/2    | —                                      | 1/1 | 1/1 | 1/1   | —   | —   | —    |
| Mikkeli .....        | 1/1   | 3/3    | 1/1                                    | —   | —   | 1/1   | —   | —   | —    |
| Kuopio .....         | 6/3   | 3/2    | 27/3                                   | —   | —   | 3/1   | 2/1 | —   | —    |
| Pohjois-Karjala .... | 2/2   | —      | —                                      | —   | —   | —     | —   | —   | —    |
| Vaasa .....          | —     | 9/5    | (No samples from the local laboratory) |     |     |       |     |     |      |
| Keski-Suomi .....    | 4/2   | 5/3    | 1/1                                    | —   | —   | 2/1   | —   | —   | —    |
| Oulu .....           | —     | 3/3    | 5/2                                    | —   | —   | 12/5  | 1/1 | 1/1 | —    |
| Lappi .....          | —     | 2/1    | —                                      | —   | —   | —     | —   | —   | —    |
| Finland, total ..... | 25/15 | 355/63 | 47/12                                  | 5/4 | 3/3 | 47/16 | 6/5 | 1/1 | 15/6 |

possible to phage type 15 of the strains. The most common phage type during the investigation period has been 1a, of which there have been 355 cases (70%). This has caused an extensive epidemic in western Finland consisting of Turku town and 31 surrounding districts. The Uusimaa (Helsinki) cases have possibly been of the same epidemic and obviously the epidemic has also appeared in the vicinity of Vaasa province of which our study has no further information. According to the investigation of veterinary surgeon Wasenius plenty of *Salmonella typhi murium* of the same phage type (Lilleengen type 8) has been found in the cattle in the area of Vaasa province. Phage type 1a can be considered to have spread all over the country as sporadic cases. The next most common have been phage types 1a var. 1 and 2b (9% and 9%). In Kuopio province phage type 1a var. 1 has appeared as an epidemic. Other detected *Salmonella typhi murium* phage types have been: 1 (5%), 2 (1%), 2a (under 1%), 2c (1%), and 3a (under 1%).

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## THE PHAGE TYPES OF SHIGELLA SONNEI

## REPORT ON 81 HUMAN CASES

by

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In the phage typing of *Shigella sonnei* strains the Hammarström method has been used. Using 12 phages (I—XII) he divides this group of bacteria into a hundred different types. The method has been obtained from the State Bacteriological Laboratory, Stockholm.

In a previous study we have shown that an extensive *Shigella sonnei* epidemic took place in Finland in 1959. The phage typing of the strains consists of the strains of 16 cases from December 1959 and the strains of 65 cases from 1960. The strains are those of cases diagnosed by the State Serum Institute whose laboratory covers the middle part of the south and the middle of Finland, the provinces of Uusimaa, Häme, and Keski-Suomi. Two strains have been obtained from Kuopio bacteriological laboratory.

THE PHAGE TYPES OF SHIGELLA SONNEI. CASES/LOCALITIES

| Year | Cases | Phage Types of <i>Shigella Sonnei</i> Strains |     |     |     |     |       |
|------|-------|---|-----|-----|-----|-----|-------|
|      |       | 11  | 39  | 58  | 73  | 79  | 85    |
| 1959 | 16    | —   | 4/1 | 1/1 | 2/1 | 3/1 | 6/5   |
| 1960 | 65    | 1/1   | —   | —   | 1/1 | —   | 63/20 |
|      | 81    | 1   | 4   | 1   | 3   | 3   | 69    |

The results of the phage typing of the *Shigella sonnei* strains of these 81 patients are shown in the table. The main part (81%) of the typed strains has been of phage type 85. The 1960 epidemic

has been almost solely caused by this type. Only two other types, one case each, have been detected: type 11 (Kuopio) and 73 (in the same district as the cases in 1959). As far as it is possible to say anything on the basis of the few typed strains of 1959, it can be presumed that also in 1959 phage type 85 has been most common and most widely spread. The epidemic, however, continuous but also other types have been detected, such as 39, 58, 73 and 79 which have appeared by districts.

In spite of the fact that the exact typing with Hammarström's method is difficult, we have been able to type the material to six different phage types, of which one has been the most common.

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